DENGUE SURVEILLANCE, REFERENCE AND RESEARCH

1981

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

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CENTERS FOR DISEASE CONTROL

CENTER FOR INFECTIOUS DISEASES

DIVISION OF VECTOR-BORNE VIRAL DISEASES

DENGUE BRANCH

SAN JUAN, PUERTO RICO 00936

AND

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1. SERVICE ACTIVITIES

1.1. Serology and Virus Isolation - Summary

As a part of the service activities to the Pan American Health Organization and individual countries in the Caribbean region, San Juan Laboratories acts as a reference laboratory, providing consultation and advice as well as reagents to persons or countries in need. Part of this service includes testing sera from suspected dengue cases. In 1981, the laboratory processed over 10,000 serum specimens, most of these from the Puerto Rico dengue 1 epidemic. However, significant numbers were from other countries. Table 1.1 shows the actual number of sera tested by hemagglutination-inhibition (HI) and complement fixation (CF) along with the number of patients and geographic origin. Over 6,500 sera from 3,709 patients were tested for dengue by either the HI or CF tests.

Virus isolation attempts were carried out on 1190 sera from 1189 patients. Again, the majority of these were from Puerto Rico. Isolation and identification was primarily by the C6/36 mosquito tissue culture-monoclonal antibody system described below. Thus, 163 viruses were isolated from 736 sera for an isolation rate of 22% compared to 213 isolates from 454 sera by the mosquito inoculation technique for an isolation rate of 47%. Overall, 376 viruses were isolated for an isolation rate of 32%.

1.2. Distribution of Dengue Viruses in Caribbean

Using the serologic and virologic results from the above tests, it has been possible to monitor dengue virus activity in the Caribbean basin. Thus, the first introduction of dengue 4 into the Caribbean was detected in April 1981 by testing sera from a tourist who had spent a holiday in the French West Indies. This person had spent the last 2 weeks of March in St. Barthelemy and became ill after returning to the United States. He had a monotypic serological response to dengue 4 and this virus serotype was isolated from the acute serum. Investigation revealed that an outbreak of dengue-like illness had begun in February of that year. Dengue 4 subsequently spread to most islands in the Caribbean.

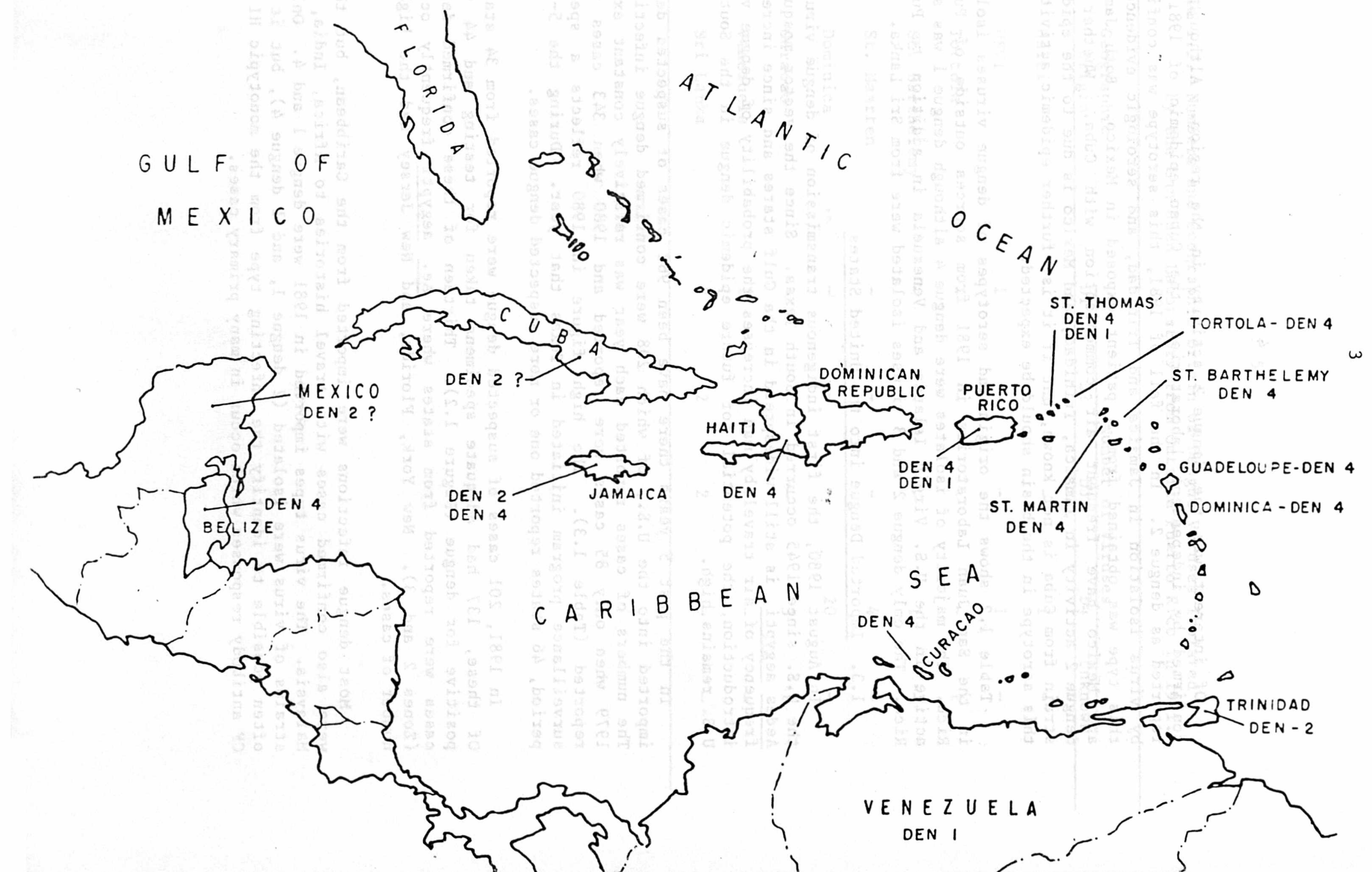
The known distribution of dengue viruses at the end of 1981 is shown in Figure 1.1. It will be noted that most of the activity in the region was due to dengue 4. Early in 1981, dengue 1 was the only known active virus in the basin, but with the introduction of dengue 4 in February or March into the French Antilles, and the subsequent epidemics throughout the Antilles, this type became the predominant virus. Later in the year, serologic and virologic evidence indicated that dengue 4 was active in Puerto Rico, Haiti, Jamaica, and Belize.

Table 1.1. Numbers of Sera Tested from Patients with Suspected Dengue, San Juan Laboratories, 1981.

Geographic Region	Number of Patients	Sera Tested HI	Number CF
Puerto Rico	2679	5035	1216
Caribbean	624	781	188
Central & South America	43	43	22
United States	304	482	298
Other	59	101	24
Totals	3709	6442	1748

FIGURE 1.1

DENGUE IN THE CARIBBEAN - 1981



Of interest was the dengue 2 activity in the region. Although not confirmed by independent laboratories, the Cuban epidemic of 1981 was reported as dengue 2. In the fall of 1981, this serotype was confirmed by virus isolation in Jamaica and Trinidad, and serologic evidence of this type was obtained from a patient exposed in Mexico. Both Jamaica and Mexico have frequent air communication with Cuba. Whether the dengue 2 activity in Jamaica, Trinidad, and Mexico is due to the epidemic strain from Cuba is not known, but if it is, further epidemic activity by this serotype in the basin should be expected.

Table 1.2 shows the origin and serotypes of dengue viruses isolated in the San Juan Laboratories in 1981 from sources outside of Puerto Rico. The majority of isolates were dengue 4 although dengue 1 was still active in the U.S. Virgin Islands and Venezuela in addition to Puerto Rico. The only dengue 2 and 3 viruses isolated were from Sri Lanka.

1.3. Imported Dengue into the United States

In August 1980, the first indigenous transmission of dengue virus in the U.S. since 1945 occurred in South Texas. Since the vector mosquito, Aedes aegypti, is still widespread in the Gulf states and since increased frequency of air travel by man increases the probability of dengue virus introduction, the potential for future epidemic dengue in the Southern U.S. remains high.

In the past 5 years there have been 962 cases of suspected dengue imported into the U.S., of which 208 were confirmed dengue infections. The numbers of cases reported each year was relatively constant except 1979 when only 85 cases were reported and 1980 when 343 cases were reported (Table 1.3). The high figure in 1980 reflects a special surveillance program initiated in Texas that year. During the 5-year period, 46 states reported one or more suspected dengue cases.

In 1981, 201 cases of suspected dengue were reported from 34 states. Of these, 137 had adequate specimens taken for testing and 44 were positive for dengue (Figure 1.2). Thirteen of these confirmed dengue cases were reported from states where Ae. aegypti frequently occurs (Zones 2 and 3). New York, Florida, and New Jersey had the highest number of cases.

Most dengue infections were imported from the Caribbean, but there were also confirmed cases with travel histories to Africa, India, and Malaysia. The virus types imported in 1981 were dengue 1 and 4. Only 3 strains of virus were isolated (2 dengue 1, and 1 dengue 4), but it was often possible to identify the infecting type from the monotypic HI and CF antibody responses which occur in many primary cases.

Table 1.2. Numbers and Geographic Origin of Dengue Viruses Isolated by the San Juan Laboratory from Outside Puerto Rico in 1981.

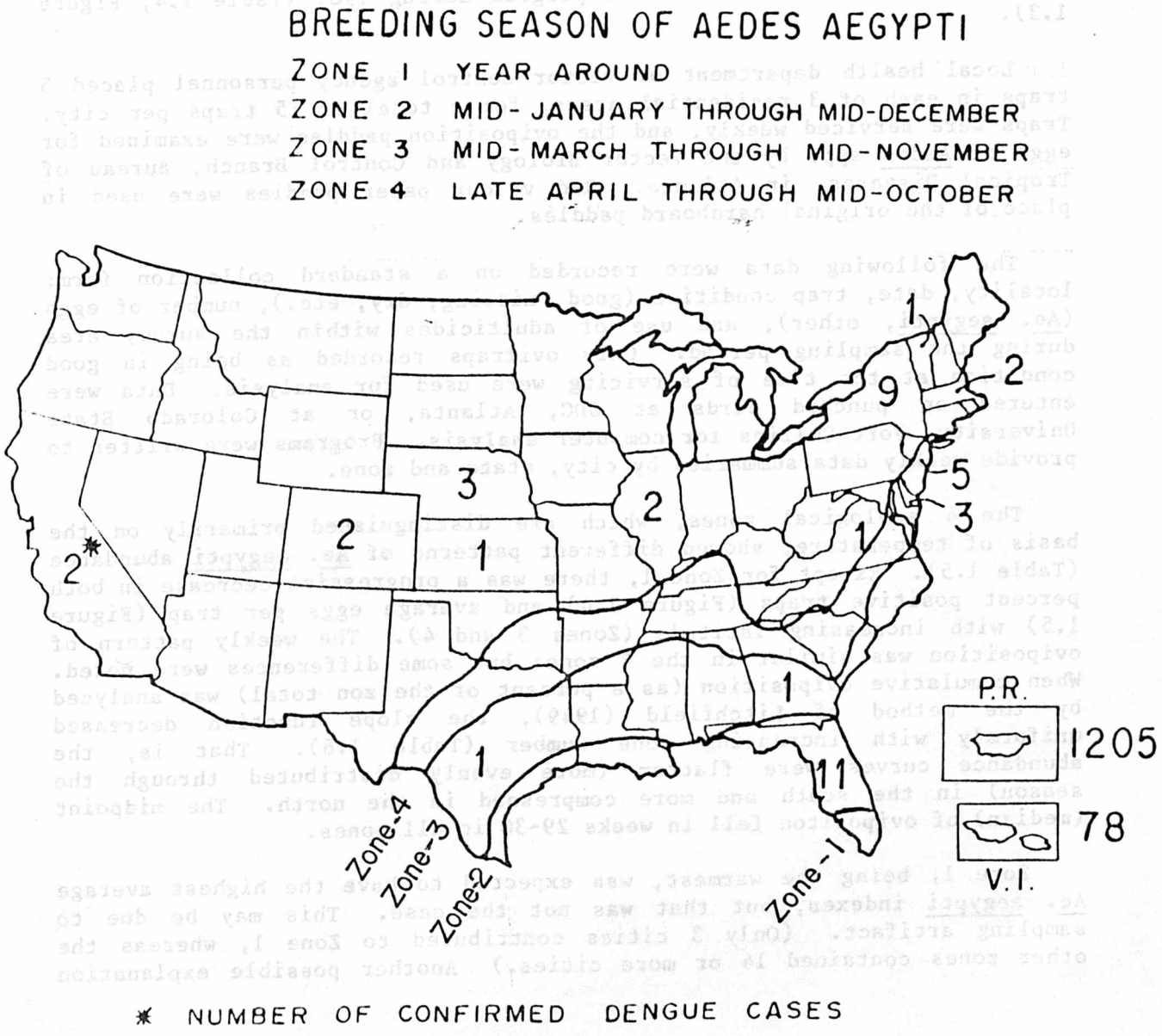
Geographic Origin	Number of Viruses				
	D1	D2	D3	D4	Unknown
Haiti	-	-	-	1	-
USVI	1	-	-	1	-
Tortola	-	-	-	-	1
St. Barthelemy	-	-	-	1	-
St. Maarten	-	-	-	4	-
Dominica	-	-	-	20	-
Curacao	-	-	-	3	1
Venezuela	1	-	-	-	-
Sri Lanka	3	2	2	-	-

Table 1.3. Imported Dengue - U.S. Mainland, 1977-1981.

Year	Total cases of suspected dengue reported	Number cases with adequate specimens	Number positive
1977	189	139	57
1978	144	100	52
1979	85	57	10
1980	343	255	45
1981	201	137	44
Totals	962	688	208

FIGURE 1.2

IMPORTED CONFIRMED DENGUE CASES, 1981, AND THE DISTRIBUTION OF *AEDES AEGYPTI* IN THE UNITED STATES



1.4. Aedes aegypti Collaborative Surveillance - United States.

A cooperative federal-state Aedes aegypti surveillance was reinitiated in 1980 in response to the threat of introduction of dengue virus into the United States from the Caribbean or from Central America. The surveillance program, which records weekly changes in Ae. aegypti density as measured by the CDC ovitrap was continued in 1981.

A total of 51 cities, representing 10 states and 4 ecological zones, participated in the surveillance program during 1981 (Table 1.4, Figure 1.3).

Local health department or vector control agency personnel placed 5 traps in each of 3 residential areas, for a total of 15 traps per city. Traps were serviced weekly, and the oviposition paddles were examined for eggs of Aedes spp. by the Vector Biology and Control Branch, Bureau of Tropical Diseases, in Atlanta. Red velour paper paddles were used in place of the original hardboard paddles.

The following data were recorded on a standard collection form: locality, date, trap condition (good, missing, dry, etc.), number of eggs (Ae. aegypti, other), and use of adulticides within the survey area during the sampling period. Only ovitraps recorded as being in good condition at the time of servicing were used for analysis. Data were entered on punched cards at CDC, Atlanta, or at Colorado State University, Fort Collins for computer analysis. Programs were written to provide weekly data summaries by city, state and zone.

The 4 ecological zones, which are distinguished primarily on the basis of temperature, showed different patterns of Ae. aegypti abundance (Table 1.5). Except for Zone 1, there was a progressive decrease in both percent positive traps (Figure 1.4) and average eggs per trap (Figure 1.5) with increasing latitude (Zones 3 and 4). The weekly pattern of oviposition was similar in the 4 zones but some differences were noted. When cumulative oviposition (as a percent of the zon total) was analyzed by the method of Litchfield (1949), the slope function decreased uniformly with increasing zone number (Table 1.6). That is, the abundance curves were flatter (more evenly distributed through the season) in the south and more compressed in the north. The midpoint (median) of ovipositon fell in weeks 29-30 in all zones.

Zone 1, being the warmest, was expected to have the highest average Ae. aegypti indexes, but that was not the case. This may be due to sampling artifact. (Only 3 cities contributed to Zone 1, whereas the other zones contained 14 or more cities.) Another possible explanation

is that Zone 1 is ecologically different from the remaining 3 zones. Southern Florida may have more in common with Caribbean areas, in which rainfall is a major controlling factor. Temperature appears to be a major limiting factor in Zones 2-4.

Summary data for the 10 participating states are shown in Table 1.7. Average eggs per ovitrap ranged from 3.4 in Tennessee to 29.2 in Arkansas. There was close agreement between average eggs per trap and percent positive traps.

Table 1.4. Collaborating cities, *Aedes aegypti* cooperative ovitrap survey, 1981.

	Zone		Zone
Alabama		**North Carolina**	
Birmingham	3	Charlotte	4
Mobile	2	Durham	4
Huntsville	4	Fayetteville	3
		Greensboro	4
Arkansas		Wilmington	3
		Winston-Salem	4
El Dorado	3	Rocky Mount	3
Little Rock	4		
Pine Bluff	3	**South Carolina**	
Texarkana	3		
		Charleston	3
Florida		Columbia	4
		Greenville	4
Daytona Beach	2	Orangeburg	4
Ft. Myers	1	Spartanburg	4
Jacksonville	2	Florence	4
Sarasota	2		
Tampa	2	**Tennessee**	
Orlando	2		
Key West	1	Chattanooga	4
Miami	1	Memphis	4
Tallahassee	2	Nashville	4
		Knoxville	4
Georgia		**Texas**	
Savannah	3	Brownsville	2
		Corpus Christi	2
Louisiana		Dallas	4
		Houston	2
Baton Rouge	3	Laredo	3
Lake Charles	2	McAllen	2
Monroe	3	San Antonio	3
New Orleans	2	Weslaco	2
Shreveport	3		
Mississippi			
Cleveland	3		
Jackson	3		
Meridian	3		
Tupelo	3		

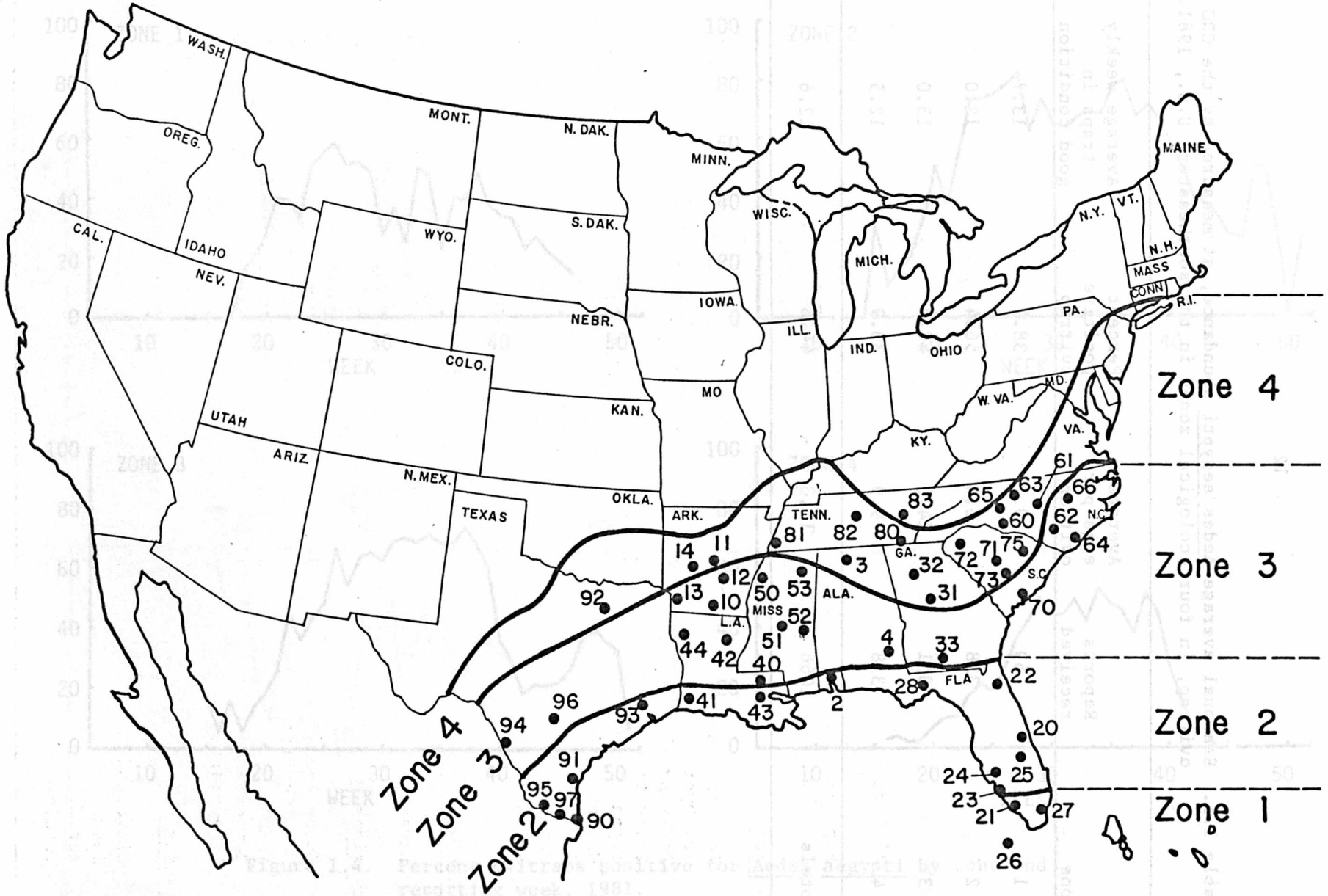
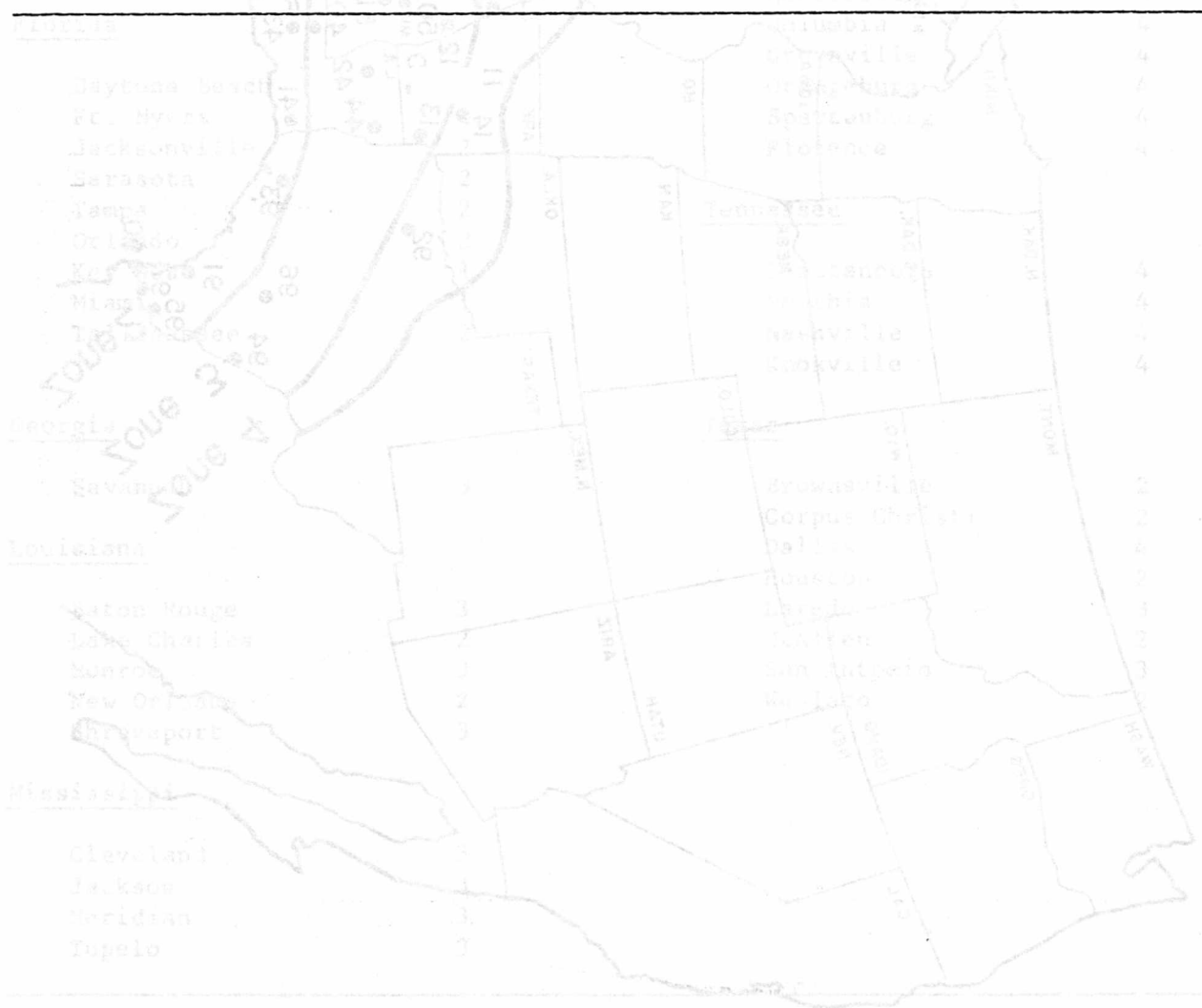


Figure 1.3. Location of cities participating in the cooperative Aedes aegypti surveillance program. Heavey lines denote boundaries of climatic zones.

Table 1.5. Seasonal average Aedes aegypti abundance, as measured by the CDC ovitrap, in four ecological zones in the southeastern U.S., 1981.

Zone	Reports received	Average eggs per ovitrap	Percent positive ovitrap	Average weekly traps in good condition
1	69	9.2	38.1	13.7
2	328	16.4	56.4	12.0
3	351	14.5	47.8	13.0
4	318	9.9	30.9	12.5
Totals	1066	13.3	44.6	12.6



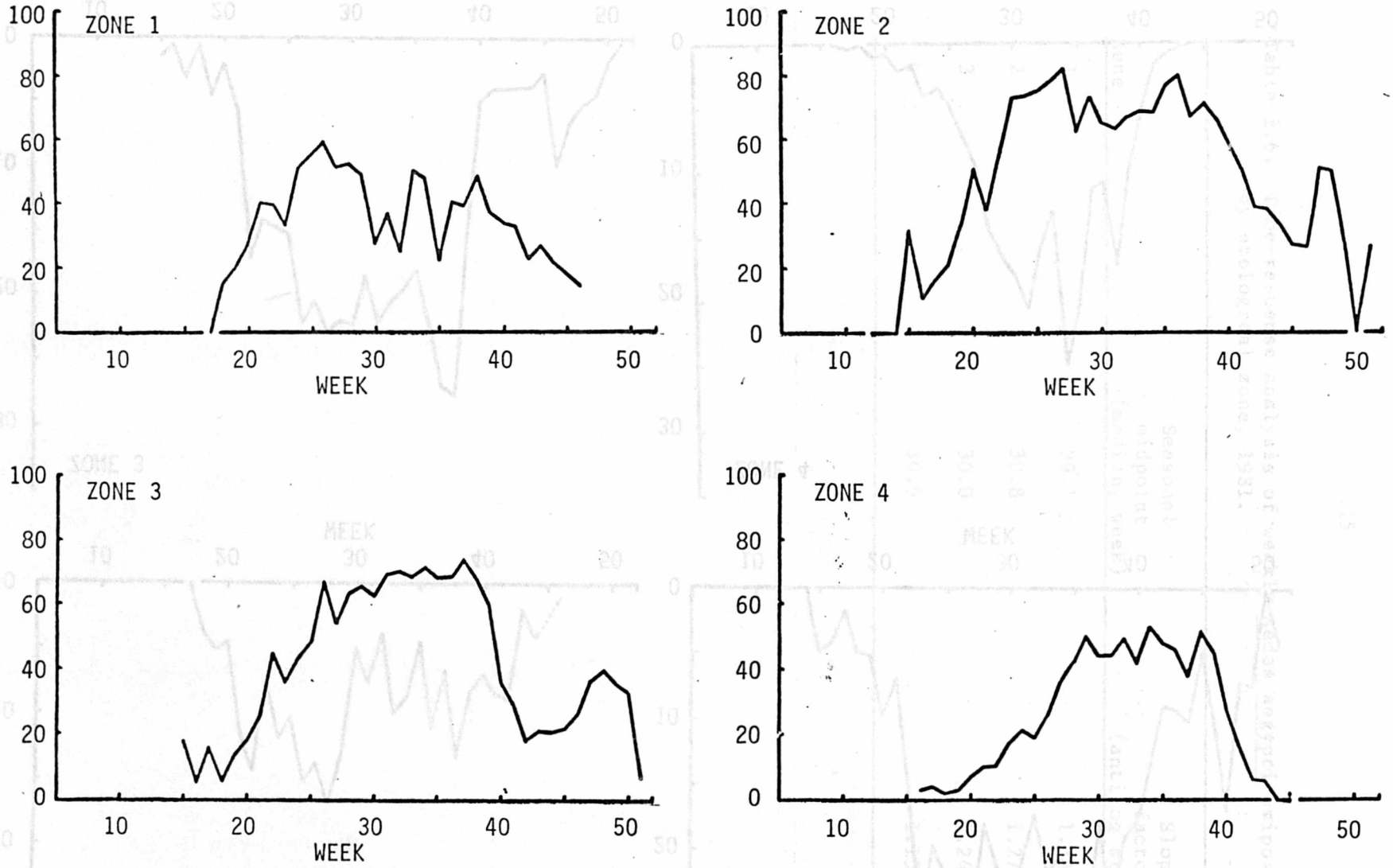


Figure 1.4. Percent ovitraps positive for Aedes aegypti by zone and reporting week, 1981.

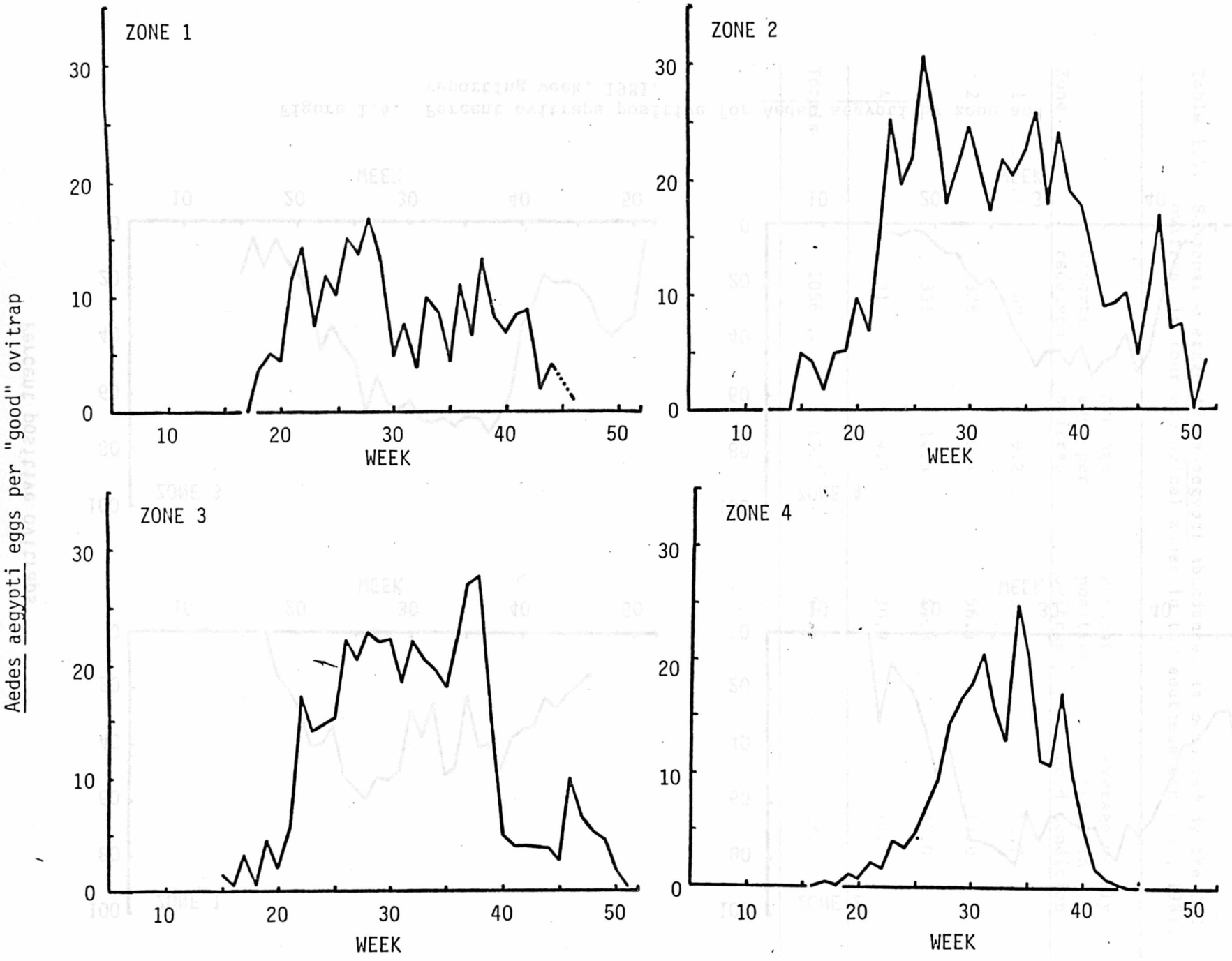


Figure 1.5. Average numbers of Aedes aegypti eggs per ovitrap, by zone and reporting week, 1981. "Good" traps are those not dried out, flooded or missing.

Table 1.6. Time-response analysis of weekly *Aedes aegypti* oviposition, by ecological zone, 1981.

Zone	Seasonal midpoint (median, week)	Slope factor (antilog std. dev.
1	29.1	1.312
2	30.8	1.270
3	30.0	1.240
4	30.9	1.151

Table 1.7. Seasonal average Aedes aegypti abundance, as measured by the CDC ovitrap, in ten southeastern states, 1981.

State	Reports received	Average eggs per ovitrap	Percent positive ovitraps	Average weekly traps in good condition
Alabama	35	14.3	56.3	9.3
Arkansas	75	29.2	65.8	13.3
Florida	196	16.2	55.2	13.0
Georgia	28	7.3	28.9	12.5
Louisiana	114	15.0	49.5	12.0
Mississippi	67	12.1	40.6	12.8
N. Carolina	176	5.3	22.6	13.2
S. Carolina	88	8.0	33.9	10.7
Tennessee	73	3.4	14.1	12.5
Texas	214	16.7	58.2	13.0
Total	1066	13.3	44.6	12.6

The incidence of unusable traps (due to drying, filling by rain, disappearance, etc.) was relatively low in most states averaging about 2-3 per week. Only Albama and South Carolina had major problems with trap loss or damage.

The cooperative surveillance program will be continued on a reduced scale in 1982. Approximately 20 cities will be asked to participate in the 1982 program.

1.5. Serological Surveys for Dengue Antibody in Haiti and Dominican Republic

Little is known about dengue transmission in Haiti and the Dominican Republic. In recent years when large epidemics occurred on neighboring islands, only sporadic cases were documented from this island. To have a better understanding of dengue activity in these countries, sera collected in 1980 were screened for dengue HI antibodies using 8 units of Dengue 1, 2, 3, and 4 antigen. Sera with antibody titers of 10 or greater were considered positive. The results, shown in Table 1.8, indicate a very high endemicity for dengue in both countries with antibody rates of approximately 70% in children under the age of 10 years. This is considerably higher than Puerto Rico and suggests that lack of dengue in Haiti and Dominican Republic is due to inadequate surveillance and reporting.

Table 1.8. Dengue HI Antibody ($\geq$10) in Persons from Haiti and Dominican Republic by age, 1980.

Age group	Haiti			Dominican Republic		
	No. tested	No. positive	%	No. tested	No. positive	%
0-10	39	28	72	154	107	69
11-20	159	110	69	61	56	92
21-30	238	220	92	-	-	-
31+	186	162	87	2	2	100
Unknown	20	20	100	127	104	82
Total	642	540	84	344	269	78

2. EPIDEMIC DENGUE IN PUERTO RICO

Puerto Rico has experienced several epidemics of dengue in recent years. In 1975-1976, dengue 2 was responsible for a large epidemic which was to a large extent, localized in the San Juan metropolitan area and the South coast. In 1977, another epidemic occurred with cases reported from the entire island. This epidemic peaked in September of that year and was apparently caused by both dengue 2 and dengue 3 as these viruses were isolated in approximately equal numbers. Although serological evidence indicated that dengue 1 was in Puerto Rico as early as August 1977, the virus was not isolated until December of that year. During the first 3 months of 1978, dengue 1 gradually replaced dengue 2 and 3 as the predominant virus. These latter serotypes were last isolated in May and March 1978, respectively. Beginning in April 1978, increased transmission of dengue 1 began with the epidemic peak occurring in June-July. All parts of the island were affected, but most of the cases were from the San Juan metropolitan area. During 1979, 1980, and the first 6 months of 1981, dengue activity was low, and dengue 1 was the only virus isolated. In July 1981, increased dengue activity was reported again. Figure 2.1 shows the epidemic curve of reported cases of dengue by week of onset. Transmission increased to a peak of nearly 800 cases per week in October and then fell off sharply during November and December. Figure 2.2 shows the distribution of confirmed dengue cases in Puerto Rico by month of onset. It will be noted that the actual peak of confirmed cases occurred in September. Beginning in October, the proportion of reported cases confirmed as dengue decreased dramatically. Overall, 52% of reported cases tested serologically or virologically were confirmed as dengue infection.

In contrast to the 1978 dengue 1 epidemic, most of the 1981 transmission of this virus serotype occurred in the south and western parts of the island. Interestingly, Fajardo, on the East coast also reported a lot of dengue activity in 1981 compared to 1978 when few cases were reported. Thus, it appears that the 1981 dengue 1 epidemic, although transmission was island wide, occurred primarily in those areas which were least affected in 1978.

Dengue virus isolations during the 1981 epidemic are shown in Figure 2.3. Dengue 1 was the only virus being transmitted in the early stages of the epidemic. Dengue 4 was introduced in September, and although confirmed cases of this serotype were sporadic in October, it was spreading on the island. In November, transmission of this serotype began to increase and by December, dengue 4 had replaced dengue 1 as the dominant virus in Puerto Rico. In contrast to the dengue 1 epidemic during the summer months, most of the dengue 4 transmission occurred in the San Juan metropolitan area. Overall, 299 dengue viruses were isolated and typed in 1981. Dengue 1 was predominant with 220 (74%) isolates compared to 79 (26%) dengue 4 isolates.

FIGURE 2.1

REPORTED DENGUE CASES BY WEEK OF ONSET PUERTO RICO - 1981

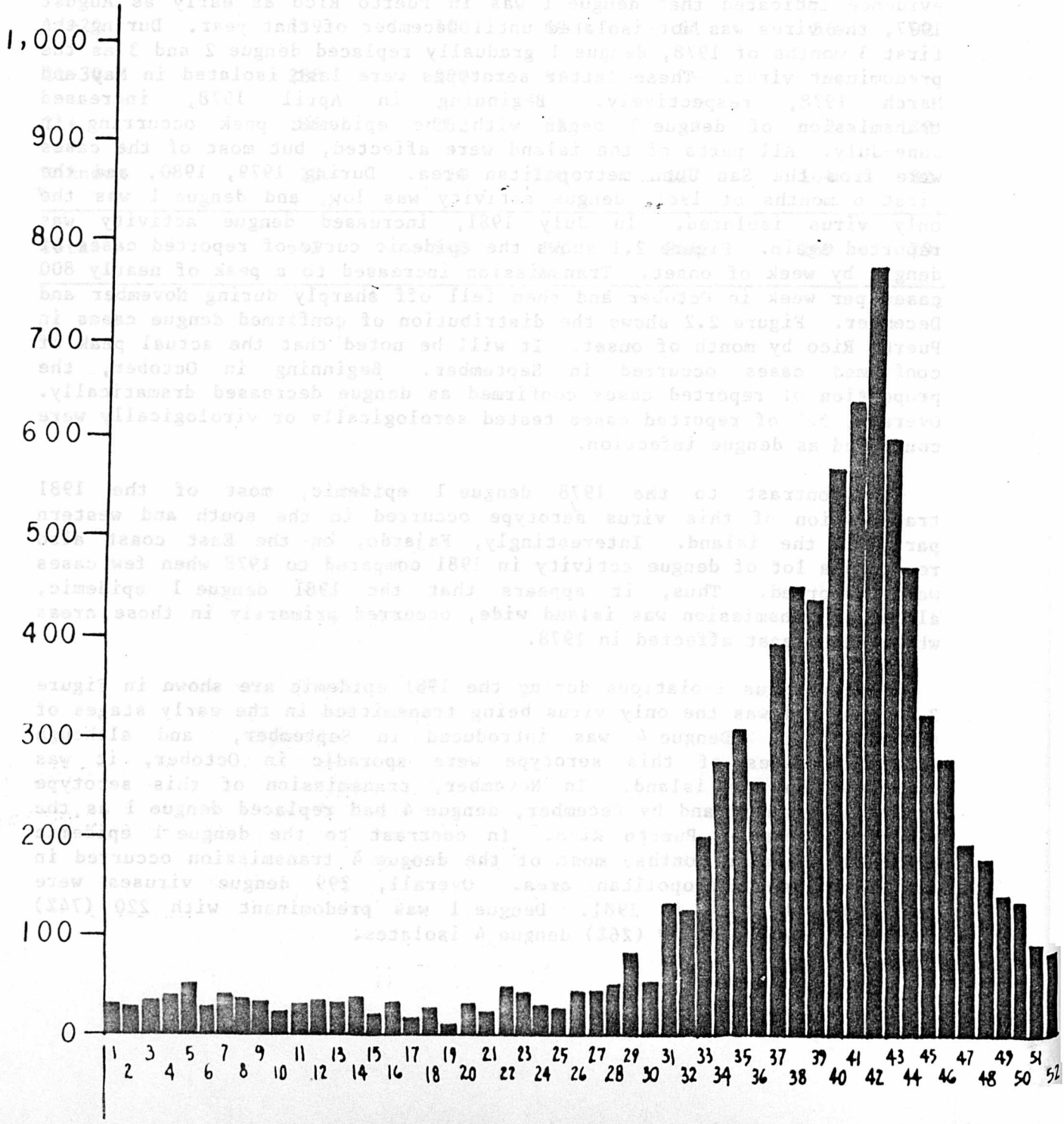


FIGURE 2.2

CONFIRMED* DENGUE CASES BY MONTH OF ONSET PUERTO RICO, 1981

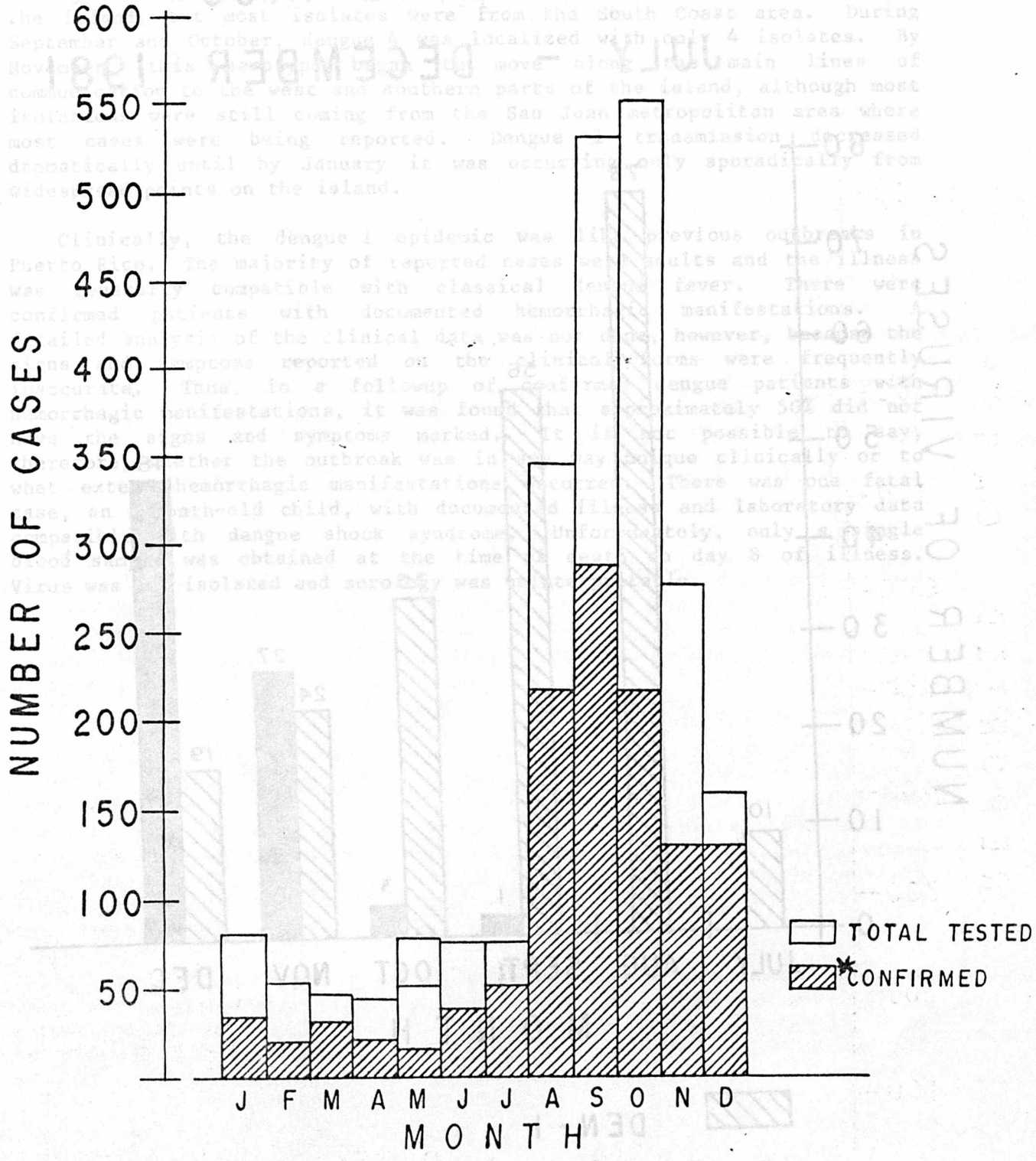


FIGURE 2.3

VIRUSES ISOLATED AND IDENTIFIED

PUERTO RICO

JULY - DECEMBER 1981

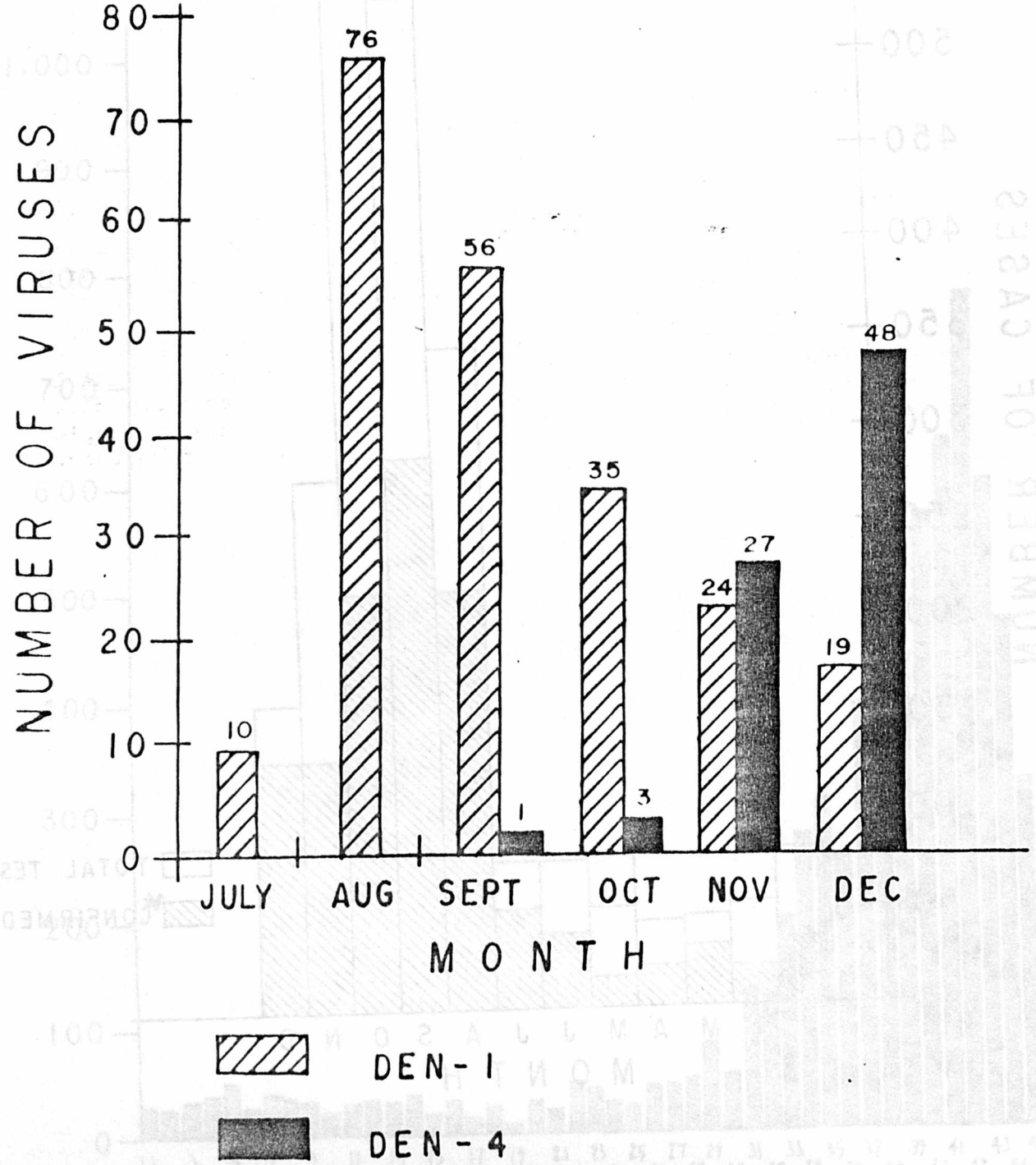
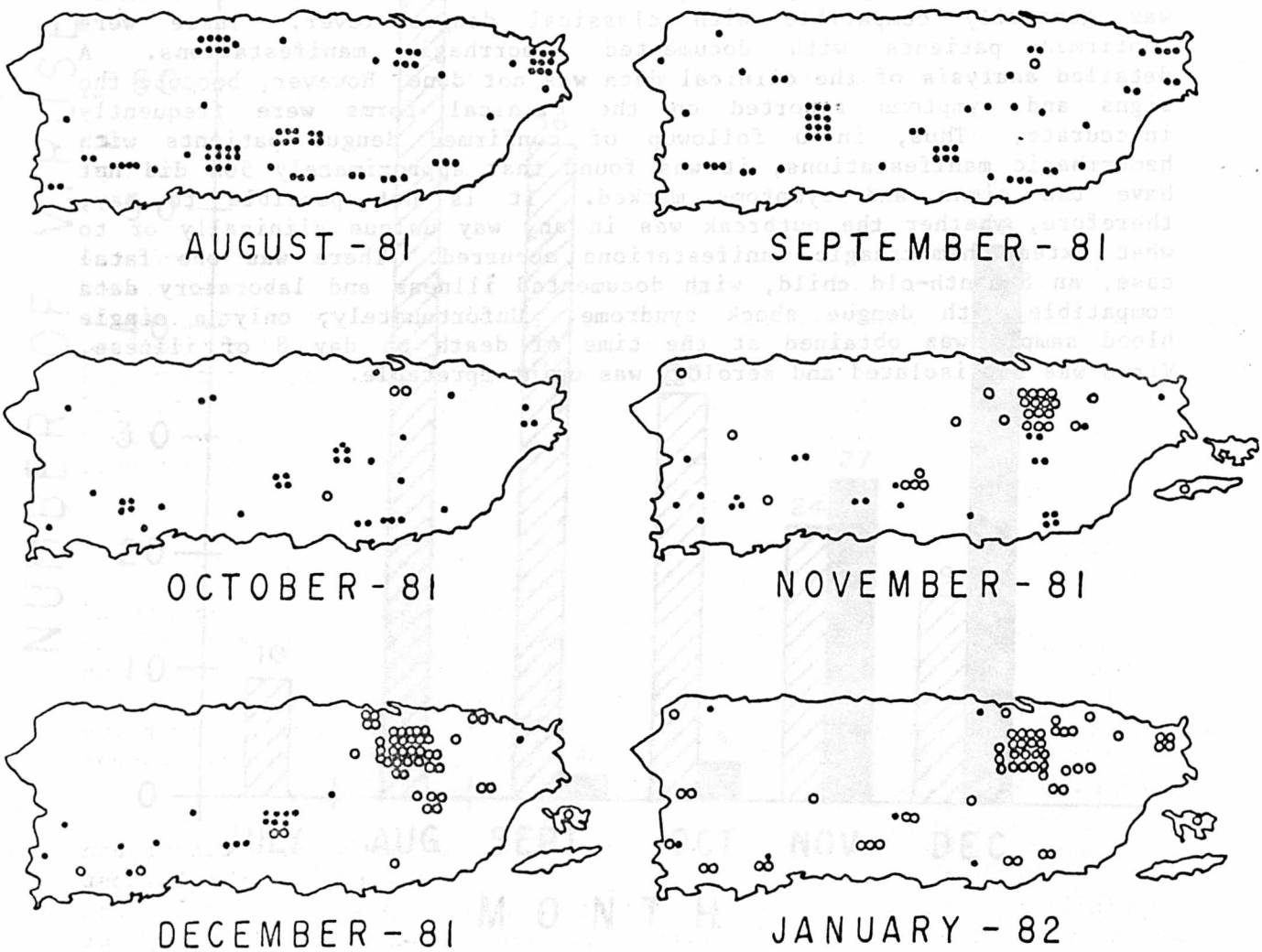


Figure 2.4 shows the distribution and movement of dengue 1 and dengue 4 viruses in Puerto Rico by month of onset. It will be noted that during the August-September period, dengue 1 was isolated from all over the island, but most isolates were from the South Coast area. During September and October, dengue 4 was localized with only 4 isolates. By November, this serotype began to move along the main lines of communication to the west and southern parts of the island, although most isolations were still coming from the San Juan metropolitan area where most cases were being reported. Dengue 1 transmission decreased dramatically until by January it was occurring only sporadically from widespread points on the island.

Clinically, the dengue 1 epidemic was like previous outbreaks in Puerto Rico. The majority of reported cases were adults and the illness was generally compatible with classical dengue fever. There were confirmed patients with documented hemorrhagic manifestations. A detailed analysis of the clinical data was not done, however, because the signs and symptoms reported on the clinical forms were frequently inaccurate. Thus, in a followup of confirmed dengue patients with hemorrhagic manifestations, it was found that approximately 50% did not have the signs and symptoms marked. It is not possible to say, therefore, whether the outbreak was in any way unique clinically or to what extent hemorrhagic manifestations occurred. There was one fatal case, an 8-month-old child, with documented illness and laboratory data compatible with dengue shock syndrome. Unfortunately, only a single blood sample was obtained at the time of death on day 8 of illness. Virus was not isolated and serology was uninterpretable.

FIGURE 2.4

DISTRIBUTION AND MOVEMENT OF DENGUE VIRUSES IN PUERTO RICO



3. FIELD STUDIES, PUERTO RICO

3.1. Background

Epidemic dengue fever, caused by all 4 serotypes, has been a major public health problem in the Caribbean region for the past 10 years. In general, hemorrhagic disease associated with these epidemics has been reported only sporadically. Recent evidence from Puerto Rico, however, indicates that severe and fatal disease which is compatible with dengue hemorrhagic fever (DHF) is occurring more frequently in the San Juan area.

Control of this disease currently is dependent upon mosquito control, usually with insecticides after the epidemic is already in progress. If the factors responsible for the distribution and spread of epidemic dengue were better known, it is possible that a predictive capability for epidemic activity could be developed and thus allow more effective preventive measures to be initiated before peak epidemic activity. The best way to identify these factors is to carry out comprehensive studies on host, vector, and virus related factors which might influence transmission in areas where epidemic and endemic activity is occurring. These studies were initiated in 1981.

The overall objective is to gain a better understanding of the ecology of dengue, thus allowing development of more efficient methods of surveillance, prevention and control of epidemic activity in the future. Specific objectives will be to identify host, vector, and virus related factors which influence transmission, and thus the distribution and spread of dengue viruses, and to clinically define the disease associated with dengue virus infection in the Americas, with emphasis on those features which may differ from the disease in Asia and on the hemorrhagic disease in primary and secondary cases.

3.2. Human Studies

3.2.1. Clinical-virological surveillance. The objective of this program is to monitor the different strains and serotypes of dengue viruses being transmitted in various parts of Puerto Rico and to correlate this with the severity of illness associated with dengue infection. This type of program will also allow us to promptly identify other viruses such as Ross River and yellow fever if they are introduced into Puerto Rico.

The program was started in 4 areas: Fajardo on the East coast; San Juan and Bayamon which represent the metropolitan area and the highest population density on the island; and Caguas, which has historically been an area of low dengue endemicity. To date, there are 9 groups of

physicians, both private and nonprivate, who are collaborating on the program. They have collectively seen 501 patients of which only about half had 2 blood samples taken. Of 225 patients tested, 161 (72%) were confirmed as dengue. Most important, however, is that reliable clinical data are available for these patients and will result in a more detailed clinical definition of dengue infection in Puerto Rico. The plan is to eventually involve most of the major urban centers of Puerto Rico in this surveillance.

3.2.2. Suspected fatal dengue hemorrhagic fever/dengue shock syndrome. The objective of this study is to develop more reliable methods for diagnosis of the severe forms of dengue infections and to develop a better understanding of the pathogenesis of severe and fatal dengue disease. In collaboration with selected pathologists, autopsies will be performed on all patients who die following a febrile illness compatible with dengue or viral encephalitis. Tissues collected will be processed for virus isolation, presence of viral antigen, and histopathology.

To date, 3 fatal cases have been studied, but none have been confirmed as dengue. One of these, an 8-month-old female was admitted to University Hospital with a history of 8 days fever, cough and vomiting. She had thrombocytopenia, a petechial rash, hematemesis, and was bleeding at the site of venipuncture and from the oral mucosa. She had perioral cyanosis and was in shock. Despite fluid therapy, plasma, and thrombocyte transfusions, the patient's condition deteriorated and death occurred approximately 9 hours after admission. A blood sample taken on day 8 of illness had no detectable dengue HI antibody and virus isolation was negative. Despite the negative results, however, this case is clinically compatible with dengue shock syndrome. Unfortunately, tissue specimens were not obtained from this patient.

The 2 other fatal cases were adult males. One, a 23-year-old male, was admitted to Caguas Regional Hospital on December 4, 1981, with a 6-day history of fever, headache, weakness, and nausea. The patient was acutely ill with purpuric lesions on the lower extremities. Admission laboratory tests showed a leukocytosis, marked thrombocytopenia, and elevated creatinine. Shortly after admission the patient developed jaundice and a clinical picture of adult respiratory distress syndrome. Despite therapy which included antibiotics, steroids, whole blood transfusions, ventilatory assistance, and resuscitation efforts, the patient expired 24 hours after admission. Tissues and serum were negative for dengue virus. A single serum sample tested by HI had antibody titers consistent with a past dengue infection. The other patient, a 35-year-old male, was admitted to Caguas Regional Hospital with a 5-day history of fever, anorexia, nausea, vomiting, and weakness on December 8, 1981. He was acutely ill, with shock, jaundice and

thrombocytopenia, and had a creatinine of 13.6. Despite dialysis and vigorous supportive and antibiotic therapy, the patient expired after 48 hours of hospitalization. Virus isolation attempts from serum and tissues were negative for dengue virus. HI antibody titers suggested past infection with dengue. Serology for leptospirosis by CDC in Atlanta was consistent with recent infection in both cases.

3.2.3. Prospective seroepidemiologic studies. The main objective of this study is to obtain baseline denominator data on human populations at risk of epidemic dengue--dengue hemorrhagic fever. The data collected will provide information on the expected frequency of primary and secondary dengue infection and will be correlated with clinical, virologic and entomologic data from the same communities. The study areas are the same as those used in the clinical-virologic and entomologic studies.

To date, blood samples have been taken from approximately 2800 6-year-old children in three urban centers (Fajardo, Bayamon and Caguas). Two 12 mm filter paper discs were saturated with finger-prick blood from each child, air-dried and stored at -20°C. One sample from each child will be screened for dengue HI antibodies. All negatives will be identified and rebled after one year. The remaining disc from the first bleeding and the second sample will then be tested as a pair to determine conversion rates for the 3 areas.

3.3. Vector Studies

3.3.1. Population ecology of Aedes aegypti. In 1967 a WHO scientific group concluded that population data on insect vectors was lacking and suggested the life budget as the best approach to obtain meaningful data relating to transmission dynamics of disease. Since that time only a few population based studies have been carried out on Ae aegypti. Furthermore, there are no published studies on Caribbean Ae. aegypti. With the recent epidemic activity of dengue 1 and 4 in the Caribbean and Puerto Rico, it is essential that field studies on the population ecology of Ae. aegypti be initiated. The purpose of this study is to define and examine both the biotic and abiotic features of the environment which influence the dynamics of field populations of Ae. aegypti and thus dengue transmission in Puerto Rico.

Three cities in Puerto Rico, Fajardo, Bayamon and Caguas, have been chosen for this study as a part of the comprehensive approach outlined above. Within each city 4 or 5 study areas have been established based on: 1) socioeconomic class of inhabitants, 2) characteristics of the environment, and 3) prior dengue virus activity.

The study is divided into 2 phases. Phase 1 includes a random house/premise survey using a modification of the data format used by the Puerto Rico Department of Health. The number of houses/premises sampled will depend on the size of the study area, but will approach 100.

Phase 2 involves a detailed quantitative study of breeding containers during which the entire contents will be examined and mosquitoes counted by species and instar. Other invertebrates will also be identified and where appropriate, further limnological analyses will be done. The number of samples per container type for each study area will be calculated using $N(Total) = (TS/mE)^2$ after Huntsberger, 1967 with N corresponding to the percent incidence of positive and potential containers from the 100 house/premise survey.

Fajardo: Phase 1

Fajardo is a city of approximately 30,000 people located on the Northeast coast of Puerto Rico. Reported dengue 1 activity in 1977-1978 was low, but in 1981 considerable dengue activity was documented. The individual study areas within the city were chosen on the basis of this information. The study areas and a brief description of socioeconomic and environmental characteristics are presented in Table 3.1.

The house/premise survey was carried out during the first 2 weeks of November 1981. During this period and prior to it, rainfall was below normal whereas temperatures were higher than normal.

The results of a 325 premise survey in the four study areas in Fajardo are shown in Table 3.2. The Florencio area had the highest container and Breteau indices with 21 and 110 respectively. The Santa Isidra area was next with a Breteau of 54 and container index of 14. The Maternillo area had a Breteau index similar to the other areas (49), but a relatively low container index (5). One explanation for this is that the residents of this area are in the lowest socioeconomic class and although there are numerous containers with water, these are actively used. The Montebrisas study site had a Breteau index of 40 and a container index of 13.

Tables 3.3 through 3.6 illustrate the potential and positive larval habitats of Ae. aegypti by container type for each study site in Fajardo. On the basis of only positive containers, each study site has its own major source of larvae; Florencio - cans and miscellaneous 1 gal.; Maternillo - miscellaneous 1-5 gal., Santa Isidra - flower pots and miscellaneous <1 gal., Montebrisas - buckets. By eliminating selected containers shown in Table 3.7, the Ae. aegypti population can be reduced by at least 70%. However, two assumptions must be made to qualify these data: <1) that the mosquitoes are Ae. aegypti, and 2) that these containers are those in which the density and potential adult productivity are the highest.

Table 3.1. Characteristics of the Study Areas in Fajardo, Puerto Rico.

Study Area	Socioeconomic Category		Environmental Characteristics
	Income	House structure	
1. Montebrisas	mid-low	concrete	urban, flat, moderate vegetation
2. Santa Isidra	mid	concrete	urban, flat, moderate vegetation
3. Florencio	low	wood	suburban, mountainous dense vegetation
4. Maternillo	low	wood	urban, coastal, moderate, vegetation

Table 3.2. The Results of a 325 Premise Survey for Container Habitats of *Aedes aegypti* within 4 Different Study Areas in Fajardo, Puerto Rico, 1981.

Study Area	No. of houses Surveyed	Total No. of containers	Containers with Water: Larvae present	Larvae absent	Total	Breteau Index	Container Index
Montebrisas I-V	100	2432	40	373	413	40.0	12.8
Maternillo	55	1873	27	510	537	49.0	5.0
Florencio	60	2534	66	254	320	110.0	20.6
Santa Isidra I-IV	110	1190	59	368	427	54.0	13.8

Table 3.3. The Potential and Positive Larval Habitats of Aedes aegypti by Container Type in Study Area, Florencia, Fajardo, Puerto Rico, 1981.

Container type	Containers with Water			Percent of Total (Positive/ potential)
	Larvae present	Larvae absent	Sum	
Animal drinking containers	4	55	59	18
Drums	4	3	7	2
Buckets	7	23	30	9
Cans	19	26	45	14
Bottles	0	58	58	18
Tires	6	5	11	3.5
Flower pots	2	15	17	5
Misc. <1 gal.	11	23	34	11
Misc. 1 - 5 gal.	7	17	24	8
Misc. >5 gal.	4	7	11	3.5
Misc. natural*	2	22	24	8
TOTAL			320	

*Includes treeholes, bromeliads, etc.

Table 3.4. The Potential and Positive Larval Habitats of Aedes aegypti by Container Type in Study Area, Maternillo, Fajardo, Puerto Rico, 1981.

Container type	Containers with Water			Percent of Total (Positive/ potential)
	Larvae present	Larvae absent	Sum	
Animal drinking containers	0	71	71	13
Drums	2	12	14	3
Buckets	1	51	52	10
Cans	4	141	145	27
Bottles	0	45	45	8
Tires	4	18	22	4
Flower pots	3	34	37	7
Misc. <1 gal.	1	75	76	14
Misc. 1 - 5 gal.	8	39	47	9
Misc. >5 gal.	2	9	11	2
Misc. natural*	2	15	17	3
Total			537	

*Includes treeholes, bromeliads, etc.

Table 3.5. The Potential and Positive Larval Habitats of Aedes aegypti by ContainerType in Study Area, Santa Isidra, Fajardo, Puerto Rico, 1981.

Container type	Containers with Water			Percent of Total (Positive/ potential)
	Larvae present	Larvae absent	Sum	
Animal drinking containers	1	30	31	7
Drums	2	6	8	1.5
Buckets	0	37	37	9
Cans	0	18	18	4
Bottles	0	7	7	1.5
Tires	5	11	16	4
Flower pots	22	83	105	25
Misc. <1 gal.	13	101	114	27
Misc. 1 - 5 gal	5	39	44	10
Misc. >5 gal	7	9	16	4
Misc. natural*	4	27	31	7
Total			427	

*Includes treeholes, bromeliads, etc.

Table 3.6. The Potential and Positive Larval Habitats of *Aedes aegypti* by Container Type in Study Area Montebrisas, Fajardo, Puerto Rico, 1981.

Container type	Containers with Water			Percent of Total (Positive/ potential)
	Larvae present	Larvae absent	Sum	
Animal drinking containers	4	54	58	14
Drums	1	3	4	1
Buckets	13	138	151	36
Cans	2	14	16	4
Bottles	7	50	57	14
Tires	3	13	16	4
Flower pots	3	44	47	11
Misc. <1 gal.	4	30	34	8
Misc. 1 - 5 gal	1	11	12	3
Misc. >5 gal	2	14	16	4
Misc. natural*	0	2	2	1
Total			413	

*Includes treeholes, bromeliads, etc.

Table 3.7. The Positive and Potential Containers within the 4 Study Areas in Fajardo which Constitue 70% or More of the Habitats of *Aedes aegypti*.

Study area	Container type	Percent of total (positive/potential)
1. Florencio	Animal drinking	28%
	Bottles	18%
	Cans	14%
	Misc. <1 gal.	11%
2. Maternillo	Cans	27%
	Misc. <1 gal.	14%
	Animal drinking	13%
	Buckets	10%
	Misc. 1 - 5 gal.	9%
3. Santa Isidra	Flower pots	25%
	Misc. <1 gal.	27%
	Misc. 1 - 5 gal.	10%
	Buckets	9%
4. Montebrisas	Buckets	36%
	Animal drinking	14%
	Bottles	14%
	Flower pots	11%

Preliminary transect data for each study area showed that 5 additional mosquito species shared the larval habitat with Ae. aegypti. These are Ae. mediovittatus, Culex pipiens quinquifasciatus, Cx. secutor, Wyeomyia sp. and Anopheles grabhamii. In some study areas. Ae. mediovittatus and Cx. pipiens quinquifasciatus population exceeded those of Ae. aegypti as the predominate mosquito species inhabiting artificial containers. Ae. aegypti surveys in Puerto Rico in 1979 and 1980 (Annual Reports Bureau Tropical Disease 1979, 1980), showed that container types such as tires and buckets, although few in number, contributed 80% or more of the potential adult population of Ae. aegypti. Further, data on both assumptions will be collected as progress on the ecological analysis of larval habitats proceeds. Similar studies are in progress or planned for other areas in which clinical-virologic and seroepidemiologic studies are being carried out.

3.3.2. Evaluation of modifications in malathion application techniques for Aedes aegypti control - A comparision of the effectiveness of malathion thermal fog and ULV applications and of malathion ULV applications with and without addition of heavy aromatic naptha (HAN) against caged adult female Aedes aegypt in in Puerto Rico. During 1979 and 1980, seven applications each of ultra low volume (ULV) and thermal fogs of malathion were made in residential areas of Puerto Rico where dengue cases had been reported. Both methods of application were made in the same residential areas on different evenings to reduce the effect of the area on final results. The mean mortality of caged mosquitoes exposed to thermal fogs in outdoor locations was slightly higher than that recorded for ULV sprays (Table 3.8), and in indoor locations the mortality obtained with thermal fogs was more than double that recorded with ULV sprays. Although the malathion dosage was greater with thermal fogs than with ULV applications, the increased volume of the thermal fog (40 gal./hour compared to 2 gals/hour with ULV) was considered to be the most likely factor contributing to their increased effectiveness.

In an attempt to improve the performance of ULV, expecially in indoor locations, tests were conducted in Puerto Rico in 1981 in which the volume of ULV applications was increased by addition of heavy aromatic naptha (HAN) in a ratio of 1 part malathion: 2 parts HAN. Comparison of mortalities obtained using caged adult Aedes aegypti exposed to malathion alone or to the malathion-HAN mixture (flow rate adjusted to provide the same dosage of malathion in both cases) are shown in Table 3.9. There was a slight increase in mortality in outdoor cages and a substantial (69%) increase in mortality in indoor cages exposed to the malathion-HAN mixture compared to resuts obtained with malathion alone.

Table 3.8. Mortality of caged female Aedes aegypti exposed to malathion ULV and thermal fog application. Puerto Rico, 1979 and 1980.

	Percent Mortality*	
	Cages Placed Outdoors	Cages Placed Indoors
ULV	66.6	31.1
Thermal Fog	79.4	66.9

* Mean % mortality from 7 applications with each type of application.

Table 3.9. Mortality of caged female Aedes aegypti exposed to malathion ULV applications with and without dilution with HAN.* Puerto Rico, 1981.

	Percent Mortality**	
	Cages Placed Outdoors	Cages Placed Indoors
Malathion alone	63.6	37.6
Malathion + HAN (1:2)	82.5	63.7

* Heavy aromatic naptha.

** Mean % mortality from 6 applications with each type of formulation.

The data presented here indicate that especially in indoor locations, malathion thermal fogs were more effective than conventional ULV malathion applications against caged adult Aedes aegypti and that the addition of HAN to malathion improved the performance of ULV applications both outdoors and indoors.

Detailed analysis of data is underway to evaluate the impact of wind velocity and position of cages in relation to their distance from the delivery point of the insecticide.

4. LABORATORY STUDIES

4.1. Susceptibility of Aedes aegypti to Oral Infection with Dengue Viruses

The distribution and spread of epidemic dengue and dengue hemorrhagic fever has been unpredictable and somewhat confusing. An explosive epidemic may occur in one area or island, while only sporadic transmission may occur in another area which appears to be ecologically identical. Variation in vector susceptibility may help to explain this distribution. Furthermore, data on vector competence will be important in developing a predictive capability for epidemic dengue. The purpose of this project is to determine whether variation in susceptibility of Aedes aegypti for dengue viruses occurs among geographic strains from the southern United States, the Caribbean, and other geographic regions.

The methods and virus used were the same as those reported previously (Vector-Borne Disease Division, CID, CDC, Annual Report 1980). Briefly, the Mexican strain of dengue 1 was used to feed newly emerged female mosquitoes on a hanging drop virus suspension consisting of equal parts virus, washed human erythrocytes and 10% sucrose. Mosquitoes were incubated for 14 days at 30°C and tested for the presence or absence of viral antigen in the brain tissue by direct fluorescent antibody technique (FA).

Most susceptibility studies are done using strains of Ae. aegypti which had been colonized from eggs or larvae collected from a single focus or from small numbers of mosquitoes. Recent evidence by several investigators has suggested that colonies started from field collected eggs may not be representative of natural populations. Furthermore, nothing is known of the variation in susceptibility existing among subpopulations of a large urban population of Ae. aegypti. To answer this question, larvae were collected from San Antonio, Texas, New Orleans, Louisiana, and Miami, Florida in October 1980. In each city, collections were made on a transect with larvae collected from 8 foci in San Antonio, 6 foci in New Orleans, and 4 foci in Miami. Each collection consisted of 3,000 to 5,000 larvae. These were taken to the laboratory, reared to adults and eggs collected for storage. It was these eggs which were hatched for use in the experiments described below.

In San Antonio the collections were made on a north-south transect. We have tested 4 of these collections or populations for oral infection with dengue 1 virus; Balcones, Lindberg Park, Harlendale and Villa Coronado. The results are shown in Table 4.1. Balcones had the lowest infection rate at 12%, and Villa Coronado the highest at 23%. Overall, 32 of 174 or 18% were infected. The differences between these populations were not significant statistically.

Table 4.1. Comparative Susceptibility of Strains of Aedes aegypti from San Antonio, Texas to Oral Infection with Dengue 1 Virus*.

Strain	Number	% Infected
Balcones	3/26**	12
Lindberg Park	7/47	15
Harlendale	8/40	20
Villa Coronado	14/61	23
TOTALS	32/174	18

* Mexican Dengue 1 - Titer of feeding suspension was approximately $10^{7.3}MID_{50}$ per ml.

** number infected/number tested.

In New Orleans, 6 collections were made on an east-west transect. Four strains, Airport, Dante, Magazine, and Almonaster, have been tested for susceptibility to dengue 1 virus (Table 4.2). Infection rates ranged from a low of 12% at the Airport, to 31% at Almonaster. This difference is also not statistically significant. Overall, 37/183 or 20% of the mosquitoes were infected.

In Miami, 4 collections were made on a north-south transect. Three of these (Perrine, Central and Goulds) have been tested with infection rates ranging from 15% in Gould to 26% in Central (Table 4.3). Again the differences are not statistically significant. Overall, 38/180 (21%) were infected. These data show rather conclusively that there are no marked differences between the Miami, New Orleans, and San Antonio Ae. aegypti with infection rates in each of about 20%, nor are there any marked differences among subpopulations within these cities. Of interest, however, were the differences between these three populations and those from Browns- ville and Corpus Christie, South Texas. Both of these strains collected in 1980, had low infection rates (Table 4.4). The differences between these and the San Antonio, New Orleans and Miami strains are highly significant ($p \leq 0.01$). New collections from Brownsville and Matamoros, Mexico were made in 1981 and susceptibility tests showed low infection rates similar to those in 1980 (Table 4.5).

Data collected to date suggest that in general, strains of Ae. aegypti from the U.S. Gulf Coast, Mexico and El Salvador had relatively low susceptibility to dengue infection. Caribbean strains, on the other hand, were consistently the most susceptible to oral infection. Because the number of mosquitoes tested was small and because all strains were not tested at the same time, a rank correlation of dengue 1 susceptibility was carried out for 20 strains tested. This takes into account the sample size tested and the variation in infection rates between tests using a control mosquito strain and gives a weighted ratio to each strain. The results are shown in Table 4.6 and confirm our previous conclusions, that the strains of Ae. aegypti from the Caribbean had the highest susceptibility while strains from Texas had the lowest.

Table 4.2. Comparative Susceptibility of Strains of Aedes aegypti from New Orleans, LA. to Oral Infection with Dengue 1 Virus.

Strain	Number	% Infected
Airport	4/33**	12
Dante	10/60	17
Magazine	10/48	21
Almonaster	13/42	31
Totals	37/183	20

* Mexican Dengue 1 - Titer of feeding suspension was approximately $10^{7.5}MID_{50}$ per ml.

** Number infected/number tested.

Table 4.3. Comparative Susceptibility of Strains of Aedes aegypti from Miami, Florida to Oral Infection with Dengue 1 Virus*.

Strain	Number	% Infection
Gould	6/41**	15
Central	17/66	26
Perrine	15/73	21
Totals	38/180	21

* Mexican Dengue 1 - Titer of feeding suspension was approximately $10^{7.2}MID_{50}$ per ml.

** Number infected/number tested.

Table 4.4. Comparative Susceptibility of Strains of Aedes aegypti from South Texas to Oral Infection with Dengue 1 Virus*, 1980.

Strain	Number	% Infected
Corpus Christie	3/46**	7
Brownsville	2/39	5
Totals	5/85	6

* Mexican Dengue 1 - Titer of feeding suspension was approximately $10^{7.5}MID_{50}$ per ml.

** Number infected/number tested.

Table 4.5. Susceptibility of Mexican, Texan, and Puerto Rican Strains of Aedes aegypti to Oral Infections with Dengue 1*, 1981.

Mosquito Strain	Number	% Infected
San Juan	15/57**	26.3
Brownsville	2/31	6.4
Matamoros	5/54	9.2

* Mexican Dengue 1 - Titer of feeding suspension was $10^{7.3}MID_{50}$ per ml.

** Number infected/number tested.

Table 4.6. Rank Correlation of Dengue 1 Susceptibility for 20 Strains of *Aedes aegypti*

Strains	No. Tested	Ratio
Villalba	24	375.41 (High)
San Juan	32	204.92
Sarasota	42	177.97
Les Cayes	42	175.41
Port Au Prince	64	153.28
New Orleans (Almanaster)	42	124.00
Miami (Central)	66	104.00
San Antonio (Villa Coronado)	61	92.00
Miami (Perrine)	73	84.00
New Orleans (Magazine)	48	72.29
San Antonio (Harlandale)	40	71.43
New Orleans (Colony)	35	70.49
New Orleans (Dante)	60	68.00
San Antonio (Lindberg Park)	47	60.00
Miami (Gould)	41	52.14
Corpus Christie	46	50.85
Montemorelos	29	49.29
New Orleans (Airport)	23	43.21
San Antonio (Balcones)	26	42.86
Brownsville	39	42.37 (Low)

4.2. Vector Genetics

The precise identification of a species transmitting a pathogen is fundamental to the study of vector-borne disease ecology. It has been well documented that many mosquito species display considerable intraspecific and geographic variation in the efficiency of disease transmission. The purpose of this study is to analyze the genetic variation within and between populations of Ae. aegypti utilizing invariant genes and to correlate these data with susceptibilty to dengue viruses.

Table 4.7 illustrates the origin of Ae. aegypti strains examined. All six strains used in the genetic analysis have been tested for susceptibility to dengue virus. The strains were started from larvae collected from 20 to 30 containers from different parts of each city.

Electrophoresis was carried out using a Pharmacia Vertical Gel System. Individual mosquitoes were homogenized to release enzymes and centrifuged to obtain a clean supernatant. An aliquot of the supernatant was then placed on gradient polyacrylimide gels each with 12 sample slots. Gels with three different gradients, 2-10%, 2-16%, and 4-40%, were used, depending on the enzyme assay. The gel with its tissue samples were then subjected for a given length of time to an electric current. After electrophoresis was completed, the gel was removed and treated with a solution that contains a specific substrate and stain for the enzyme product to be assayed. The specific buffer system and histochemical straining procedures used were either those of Steiner and Joselyn (1979) or Munstermann (1978).

Gene frequencies, heterozygosities and chi-square deviations from expected Hardy-Weinberg equilibria were calculated with the VS-Basic computer program provided by Leonard Munstermann. Genetic distances between strains were generated by a Fortran computer program following the procedure of Nei (1972). The following enzymes were studied: malate dehydrogenase (MDH), octanol dehydrogenase (ODH), isocitrate dehydrogenase (IDH), phosphoglucomutase (PGM), lactate dehydrogenase (LDH), and supraoxide dimutase (SDM).

Two inbred colony strains of Ae. aegypti, one which had previously shown high susceptibility (Jakarta) and one which had previously shown low susceptibility (Shimba Hills) to oral infection with dengue viruses, were examined electrophoretically. The three loci exhibiting differentiation are shown in Table 4.8. Phosphoglucomutase, lactate dehydrogenase, and supraoxide dimutase were monomorphic and are not included in the table.

Clearly, no single locus can be used to distinguish these two mosquito strains. Contingency chi-square tests show significant strain

Table 4.7. Populations of *Aedes aegypti* Examined for Genetic Variation.

Strain	Location	Stage collected
Juan-L	Rio Piedros, P.R.	Larvae
Villa-L	Villalba, P.R.	Larvae
Quad-L	San Salvador, El Sal.	Larvae
New Orleans-L	New Orleans, LA	Larvae
Sara-F	Sarasota, FL	Eggs
Brown-L	Brownsville, TX	Larvae

variation in genotype frequencies for ODH and IDH loci (X^2 = 87.4, $P < 0.005$ and 8.4, $P < 0.025$, respectively). Furthermore, Nei's genetic index (0.633) shows a considerable amount of genetic variation between these two strains of Ae. aegypti. This is not unexpected since it is possible that the Shimba Hills strain originated from Ae. aegypti formosus (Tabachnick and Munstermann personal communication) and only six enzymes were studied. Furthermore, the frequencies agree with previous studies of these populations by Tabachnick.

Six strains from the Southern United States and Caribbean have also been examined electrophoretically to determine genetic variation. Table 4.9 shows results from the four most differentiated loci of the six strains examined. Continguency chi-square tests were used to test for significant genetic differentiation at each locus. As noted above for the Jakarta and Shimba Hills strains, no single locus can be used to differentiate these strains of Ae. aegypti. However, there appears to be genetic affinities between the Puerto Rican and Florida strains and between the New Orleans, Brownsville, and El Salvador strains.

These associations are supported by calculating Nei's index of genetic distance shown in Table 4.10 and illustrated in Fig. 4.1. The Puerto Rican strains were very similar with a genetic distance of only 0.002. These strains showed considerable genetic distance (0.206 and 0.214), however, from the El Salvador Ae. aegypti. It will be noted that the New Orleans, Brownsville, and El Salvador Ae. aegypti are more closely related to one another with genetic distances of only 0.094, 0.058, and 0.114. The Sarasota Ae. aegypti appear to be intermediate between the Puerto Rican and New Orleans strains.

Table 4.8. Frequency of Electromorphs Produced by the Variable Enzyme loci in Two Strains of *Aedes aegypti* Showing High and low Susceptibility to Oral Infection with Dengue Viruses.*

Enzyme	Band (Electromorph)	Electromorph Frequency	
		Jakarta, Indonesia	Shimba Hills, East Africa
	N+	137	154
Isocitrate Dehydrogenase	100	0.876	0.779
	108	0.109	0.221
	110	--	--
	114	0.015	--
	N	243	192
Malate Dehydrogenase	96	0.235	0.240
	100	0.346	0.354
	110	0.177	0.167
	113	0.210	0.161
	115	0.016	0.042
	Null	0.016	0.036
	N	213	167
Octanol Dehydrogenase	100	0.286	0.671
	110	0.376	0.329
	113	0.329	--
	Null	0.009	--

* Results are based on homogenates of 118 to 120 mosquitoes of each strain for each enzyme tested.

+ Number of genomes scored.

Table 4.9. Frequency of Electromorphs Produced by the Variable Enzyme loci in 6 Representative Populations of Aedes aegypti.

Enzyme*	Band (Electromorph)	Electromorph Frequency					
		San Juan	Villalba	El Salvador	New Orleans	Sarasota	Brownsville
MDH	N+	(170)	(198)	(180)	(154)	(232)	(292)
	96	0.643	0.608	0.589	0.364	0.371	0.301
	100	0.357	0.392	0.411	0.636	0.496	0.607
	110	--	--	--	--	0.066	0.038
	113	--	--	--	--	0.069	0.054
ODH-1	N	(120)	(118)	(192)	(148)	(86)	(186)
	100	1.00	0.947	0.724	0.445	0.425	0.783
	110	0.00	0.053	0.258	0.555	0.575	0.217
IDH-2	N	(190)	(124)	(148)	(148)	(156)	(178)
	100	0.482	0.466	0.486	0.743	0.442	0.402
	108	0.518	0.534	0.514	0.257	0.558	0.598
PGM	N	(84)	(86)	(100)	(100)	(84)	(100)
	100	0.985	1.000	0.397	0.563	1.000	0.188
	104	0.000	0.000	0.603	0.437	0.000	0.812
	Other	0.015	--	--	--	--	--

*Malate dehydrogenase (MDH), Octanol dehydrogenase (ODH), Isocitrate dehydrogenase (IDH) and Phosphoglucomutase (PGM).

+N = number of genomes scored.

Table 4.10. Genetic Distances Between Populations of *Aedes aegypti* in the U.S., Central America and the Caribbean.

	San Juan	Villalba	El Salvador	New Orleans	Sarasota	Brownsville
San Juan	--					
Villalba	0.002	--				
El Salvador	0.214	0.206	--			
New Orleans	0.320	0.290	0.114	--		
Sarasota	0.132	0.146	0.246	0.174	--	
Brownsville	0.396	0.382	0.058	0.094	0.520	--

FIGURE 4.1

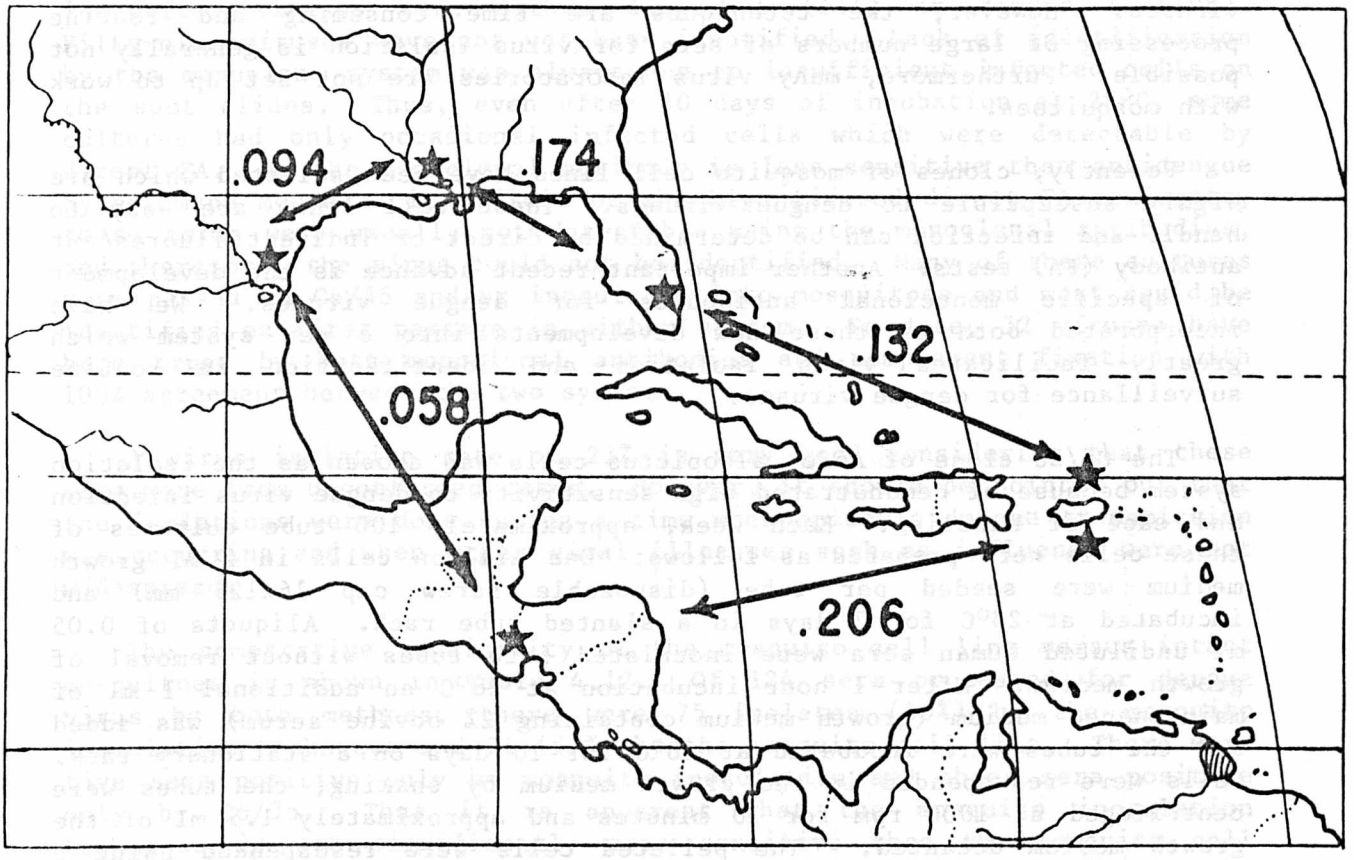


Figure 4.1. Geographic distribution of genetic distances between populations of Aedes aegypti in the United States, Central America and the Caribbean.

4.3. Mosquito Cell Lines for Primary Isolation of Dengue Viruses

4.3.1. Use of mosquito cell lines and monoclonal antibody for routine surveillance of dengue viruses. Of the important arbovirus diseases of man, dengue viruses are among the most difficult to detect and propagate in the laboratory. Development of the mosquito inoculation technique for isolation and the complement fixation test using antigen prepared in mosquitoes for identification, provided for the first time a highly sensitive method for isolation and identification of these viruses. However, the techniques are time consuming and routine processing of large numbers of sera for virus isolation is generally not possible. Furthermore, many virus laboratories are not set up to work with mosquitoes.

Recently, clones of mosquito cell lines have been selected which are highly susceptible to dengue viruses. These cell lines are easy to handle and infection can be determined by direct or indirect fluorescent antibody (FA) tests. Another important recent advance is the development of specific monoclonal antibodies for dengue viruses. We have incorporated both of these new developments into a new system which greatly facilitates virus isolation and identification in routine surveillance for dengue viruses.

The C6/36 clone of Aedes albopictus cells was chosen as the isolation system because of demonstrated high sensitivity to dengue virus infection and ease of handling. Each week, approximately 100 tube cultures of these cells were prepared as follows: One million cells in 2 ml growth medium were seeded per tube (disposable screw cap 16x125 mm) and incubated at 28°C for 3 days in a slanted tube rack. Aliquots of 0.05 ml undiluted human sera were inoculated into tubes without removal of growth medium. After 1 hour incubation at 28°C an additional 1 ml of maintenance medium (growth medium containing 2% bovine serum) was added and the tubes were incubated at 28°C for 10 days on a stationary rack. Cells were resuspended in the growth medium by shaking, the tubes were centrifuged at 1000 rpm for 10 minutes and approximately 1.5 ml of the growth medium decanted. The pelleted cells were resuspended using a pasteur pipette and spotted onto 2 slides. The remaining cells and media were stored at -70°C. On the first (FA) slide, cells were placed in only 3 wells while on the second identification (ID) slide, cells were placed in all 12 wells. After air drying, the slides were fixed in cold acetone for 10 min. and processed for FA immediately or stored frozen at -70°C until use. The presence or absence of flavivirus antigen on FA-slides was determined by direct FA using an FITC labeled conjugate prepared from high titered anti-dengue human serum. The identity of the agent was established on the corresponding ID-slide using an indirect FA with monoclonal antibodies specific to each of the 4 dengue serotypes.

This new system was begun on a routine basis in November 1981. Each week, approximately 75 acute sera from patients with suspected dengue were selected on the basis of day of illness (usually day 3 or less), symptomatology and location of residence on the island of Puerto Rico. Sera were selected and inoculated without reference to serological results.

To date, 1587 sera have been processed for virus isolation in this manner and 368 dengue viruses have been isolated (Table 4.11). All identified viruses were either dengue 1 (111) or dengue 4 (198). Fifty-nine viruses have not yet been identified. Lack of identification by the monoclone system was always due to insufficient infected cells on the spot slides. Thus, even after 10 days of incubation at 28°C, some cultures had only occasional infected cells which were detectable by direct FA. As the monoclonal antibody is less sensitive than antidengue hyperimmune mouse ascitic fluid or the high titered direct FA conjugate, these cells were usually not detectable using the monoclonal antibodies, and therefore, the virus could not be identified. Many of these cultures were passed in C6/36 and/or inoculated into mosquitoes and most could be identified on first passage in either system. To date, 32 viruses have been typed by both monoclonal antibodies and complement fixation with 100% agreement between the two systems.

A virus isolation rate of 23% is very good considering that these sera were from unconfirmed cases. However, it should be pointed out that the isolations were done during a time when epidemic dengue transmission was occurring and when other viral illnesses such as influenza were not widespread.

The comparative sensitivity of the mosquito cell line versus intact mosquitoes is shown in Table 4.12. Of 124 sera processed for dengue virus by both methods, there were 75 isolates (60%) by the mosquito inoculation technique and 71 (57%) by the mosquito cell line. There were five sera positive only by mosquito inoculation and three sera positive only by C6/36. Thus it is apparent that the mosquito inoculation technique is not significantly more sensitive than the mosquito cell lines. Furthermore, the ease with which large numbers of sera can be processed for virus isolation with limited resources makes the mosquito tissue culture-monoclone system ideal for a virologic surveillance.

Table 4.11. Dengue Virus Isolation and Identification Using C6/36 Tissue Culture Cells and Monoclonal Antibodies.

Number Sera	Number Virus Isolates						
Tested	D1	D2	D3	D4	Unknown	Total	%
1587	111	0	0	198	59	368	23

Table 4.12. Comparative Dengue Virus Isolations from Human Sera Using Mosquito Inoculation and C6/36 Tissue Culture Cells.

	Mosquito Inoculation	C6/36 Cells
Number of sera tested	124	124
Number viruses isolated	75	71
Isolation Rate	60.5	57.3

4.3.2. Comparative sensitivity of three mosquito cell lines for the isolation of dengue viruses. The use of specific monoclonal antibodies has greatly facilitated the identification of dengue viruses. Although this technique can be used to identify dengue viral antigen in mosquito brain squashes, it requires a minimum of 6 and preferably 12 known positive mosquitoes. This means that most isolates must be passed in mosquitoes at least once after isolation and thus delays the identification process, whether by monoclonal antibody or complement fixation. Although slightly less sensitive, the use of mosquito cell lines to isolate dengue viruses and the subsequent use of monoclonal antibodies to type the viruses is much less labor intensive and allows processing of larger numbers of samples on a routine basis.

These procedures were initiated during the dengue epidemic of 1981 in Puerto Rico. As a part of this transition, three mosquito cell lines, Igarashi's clone C6/36 of Aedes albopictus, Ae. pseudoscutellaris (AP-61), and Toxorhynchites amboinensis (TRA-284) were compared for sensitivity to dengue virus and ease of handling. Cells were grown in either disposable tubes (16 x 125 mm) or in plastic flasks (25 cm^2) and simultaneously inoculated with 0.05 ml each of undiluted sera collected from patients in acute phase of dengue-like illness. After 10-day incubation at 28°C, the cells were spotted on slides, fixed with cold acetone, and processed for virus isolation and identification using a direct fluorescent antibody test (DFA) for screening and indirect (IFA) with monoclonal antibodies for identification.

The results obtained with 83 sera are shown in Table 4.13. The AP-61 and TRA-284 lines were most sensitive with 31 and 29 isolates respectively. Only 25 isolates were obtained with the C6/36 cells. It will be noted that some of the viruses isolated in C6/36 and AP-61 cells could not be typed. This was due to the small number of cells infected and the small amount of antigen detectable by DFA, but not by the monoclonal IFA. These sera have been inoculated into mosquitoes for confirmation.

In addition to virus isolation rate, the three cell lines were compared with respect to the following criteria: (1) ease of handling and cultivation; (2) brightness of fluorescence; (3) resistance to toxicity of sera; (4) growth rate in different types of culture vessels; and (5) cost/culture/specimen. While ease of cultivation was nearly the same for all three cell lines, Ae. albopictus cells (C6/36) were the best in terms of uniform dispersal of cells (without clumping) on spot slides. Intensity of fluorescence was similar for all cell lines, but easier to read in C6/36 because the cells were never disrupted. TRA-284 and AP-61 cells were generally more resistant to serum toxicity than Ae. albopictus cells. While both Ae. albopictus and AP-61 cells grew well on glass as well as plastic surface, TRA-284 cells did not grow well in glass tubes. The cost of the glass tube culture was far less expensive

Table 4.13. Comparative Sensitivity of Three Mosquito Cell Lines For Isolation of Dengue Viruses in Puerto Rico.

Cell line	No. sera isolated	Number and types of dengue isolates D1	D4	Unknown	Total
C6/36	83	5	16	4	25
AP-61	83	8	21	2	31
TRA-284	83	9	20	0	29
Totals (All Cell Lines)	83	9	26	2	37

than that of plastic culture. These advantages and disadvantages will be evaluated for the selection of a cell line for routine dengue virus isolation/identification.

4.3.3. Development of new mosquito cell lines. Although Ae. aegypti is the principal vector of dengue viruses, most cell lines isolated from this species have been found to be non-sensitive to these viruses. Recently, it was reported that sensitivity to dengue viruses varies among geographic strains of Ae. aegypti. Although the use of a dengue- susceptible strain of Ae. aegypti as a source of seed cells in primary culture does not necessarily guarantee isolation of virus-sensitive cell lines, it is nevertheless of interest to isolate cell lines from a colony of Ae. aegypti, established in Puerto Rico where dengue has been endemic for many years, which may imply higher in vivo sensitivity to the viruses.

The cuticle of the larvae was ruptured using a sterilized glass rod containing fine longitudinal grooves at the tip. Extensive injury to internal organs was kept to a minimum. The damaged, but living larvae were pooled and used as sources of seed cells in primary culture. The same technique was previously found to be effective for isolating cell lines from Toxorhynchites amboinensis, but had not been tested for a smaller mosquito, such as Ae. aegypti. Unlike Tx. amboinensis, however, the mortality of Ae. aegypti larvae due to the mechanical damage exceeded 90%. This high mortality was attributed to smaller larval size of Ae. aegypti which makes it difficult to rupture the cuticle only without damaging internal organs. Furthermore, the beneficial, additive effect of mechanically damaged, but living larvae for faster and more efficient isolation of continuous cell lines from Tx. amboinensis, was not observed in case of Ae. aegypti. Nevertheless, 4 cell lines were isolated from 94 tubes containing up to 25 mechanically damaged, but living larvae per ml per tube. They were designated AGY 101, AGY 104, AGY 109, and AGY 502.

A chromosome analysis of the first 3 cell lines revealed that they were predominantly composed of diploid (2N=6) cells. With a multiplicity of infection of 0.1 PFU/cell, 4 dengue serotypes (DEN 1, Hawaii; DEN 2, New Guinea "C"; DEN 3, H-87; DEN 4, H-241) replicated in the AGY-101 cell line to extracellular titers exceeding 5 dex PFU/ml in 9 days. The Puerto Rico DEN 3 (PR-6) titers ranged between 3 and 5 dex PFU/ml. No CPE was induced by the viral infections. Morphologically, these cell lines were quite different from other Ae. aegypti cell lines, such as ATP-10 of Singh, AA-20 of Varma and Pudney, AA-20A of Varma and Pudney, and RML-12 of Bhat.

4.3.4. Adaptation of a mosquito cell line to serum-free media - effects on sensitivity to dengue virus infection. For most mosquito cell cultures, bovine sera are an indispensable ingredient of growth media. However, those bovine sera, fetal bovine sera (FBS) in particular, are expensive and have become scarce at one time in the past several years. Furthermore, bovine sera have often been incriminated in contaminating cell cultures with mycoplasma and viruses despite the fact "mycoplasma and virus-free" products were used. Therefore, the use of serum-free media is desirable, provided that these media are inexpensive and do not modify the beneficial traits of cell cultures.

For arbovirologists, often the beneficial trait in question is the sensitivity to virus infection. A popyploid cell line from Toxorhynchites amboinensis, TRA-284, was adapted to a medium consisting of L-15 and tryptose phosphate broth (TPB), and the resultant subline was designated TRA-284-SF. The SF subline, the parent TRA-284, the C6/36 clone of Ae. albopictus and LLC-MK_2 cells were simultaneously infected with the following laboratory- adapted strains of dengue viruses: DEN 1, Hawaii; DEN 2, New Guinea "C"; DEN 3, PR-6; DEN 3, H-87, and DEN 4, H241. After a 9-day-incubation period at 28°C for mosquito cells, and at 35°C for LLC-MK_2 cells, supernatant fluids were harvested. Initial inocula and extracellular virus titers in the supernatant fluids were plaque-assayed on LLC-MK_2 cell cultures. The results are shown in Table 4.14. It is apparent that the sensitivity of the TRA-284-SF cells was not significantly altered from that of TRA-284 cells. Both Toxorynchites cell lines were comparable in sensitivity with Ae. albopictus (C6/36) cells. The sensitivity of LLC-MK_2 cells was, in general, lower than that of mosquito cells. Table 4.15 shows that the TRA-284 and TRA-284-SF cells are superior to the Ae. albopictus (C6/36) cells for isolation of unadapted dengue strains. This comparison was not completely satisfactory, however, because many sera were toxic to the Ae. albopictus cell line. This may explain the lower virus isolation in that cell line. Nevertheless, in a separate, comparative study devoid of serum toxicity problem, it was clearly shown that the TRA-284 cell line was superior to Ae. albopictus cells for dengue virus isolation (see Section 4.3.2 above).

The relationship between the amount of virus in sera and virus yield 9 days after inoculation is shown in Table 4.16. Generally, higher doses in inocula resulted in a higher virus isolation rate and higher virus yield. It is of interest to note that many virus strains were recovered in high titers ($>10^3$ PFU/ml) in the TRA-284-SF cells from the specimens that did not contain demonstrable plaquing agent in the inocula. In LLC-MK_2 cells, on the other hand, virus was usually not isolated from the inocula with no PFU. Ten sera induced syncytia in the TRA-284-SF cell cultures. The inducation of syncytia was apparently a function of virus dose, since 9 out of 10 sera inducing syncytia contained 14 or more PFU per inocula, while only one serum containing no PFU per inoculum induced syncytia.

Table 4.14. Comparative Replication of Laboratory-Adapted Strains of Dengue Viruses in *Toxorhynchites amboinensis*, *Aedes albopictus*, and LLC-MK_2 Cell Cultures.

Virus	Virus Titer (Log PFU/ml supernatant)[a/] in:			
	TRA-284[b/]	TRA-284-SF[c/]	A. albopictus[d/]	LLC-MK_2
DEN 1 (Hawaii)	6.8(6.4-7.2)[e/]	7.4(6.9-7.8)[e/]	7.7(6.5-8.2)[e/]	6.4(6.3-6.6)[f/]
DEN 2 (NG"C")	7.3(6.8-7.7)	7.5(7.3-8.3)	7.7(7.0-8.3)	6.5(6.4-6.7)
DEN 3 (PR-6)	4.4(3.3-6.1)	5.4(5.0-6.0)	4.5(3.4-5.3)	4.1(4.0-4.3)
DEN 3 (H-87)	7.2(7.0-7.7)	7.3(6.8-7.8)	6.0(5.3-7.0)	6.2(6.1-6.3)
DEN 4 (H-241)	6.6(6.1-7.1)	6.4(6.5-7.3)	6.5(6.1-6.7)	6.1(6.0-6.3)

a/ Harvested on day 9 post inoculation.

b/ *Toxorhynchites amboinensis* (TRA-284) cell line.

c/ A subline from TRA-284 cells adapted to a serum-free medium.

d/ Igarashi's clone C6/36.

e/ Geometric means of 4 tests. The numbers in parentheses indicate ranges.

f/ Geometric means of 2 tests. The numbers in parenthesis indicate ranges.

Table 4.15. Comparative Dengue Virus Isolation from Human Sera in Toxorhynchites and LLC-MK_2 Cell Cultures.

Dengue Serotype [a/]	No. Sera Tested [b/]	No. Virus Strains Inoculated in: TRA-284 Cells	TRA-284-SF Cells	LLC-MK_2 Cells
DEN 1	28	25	22	10
DEN 2	6	6	6	6
DEN 3	6	2	4	1
DEN 4	8	5	4	3

a/ DEN 1, 2, 3, and 4 viruses originally isolated by intrathoracic inoculation of adult mosquitoes and identified by complement fixation at the San Juan Laboratories.

b/ One serum was extremely toxic to both cell lines, destroying all cells before the end of 9-day incubation period.

Table 4.16. Relationship Between the Amounts of Dengue Virus in Human Sera and Virus Yields in the Supernatant Fluids of Toxorhynchites and LLC-MK_2 Cell Cultures.

Dengue Serotype	Amount Virus/Inoculum (PFU/0.1 ml Serum) per Flask	No. Sera Tested	No. sera that Produced Level of Virus Yield (PFU/ml) Supernatant	Virus in Amount corresponding to the level of Virus Yield in: TRA-284-SF	LLC-MK_2
DEN 1	0	17	0	5	13
			$1 - 10^3$	6	3
			$>10^3$	6	1
	1 - 100	8	0	1	5
			$1 - 10^3$	1	2
			$>10^3$	6	1
	>100	3	0	0	0
			$1 - 10^3$	1	2
			$>10^3$	2	1
DEN 2	1 - 100	4	0	0	0
			$1 - 10^3$	0	4
			$>10^3$	4	0
	>100	2	0	0	0
			$1 - 10^3$	1	1
			$>10^3$	1	1
DEN 3	0	2	0	0	2
			$1 - 10^3$	1	0
			$>10^3$	1	0
	1 - 100	4	0	2	3
			$1 - 10^3$	1	1
			$>10^3$	1	0
DEN 4	0	4	0	2	4
			$1 - 10^3$	1	0
			$>10^3$	1	0
	1 - 100	4	0	2	1
			$1 - 10^3$	0	2
			$>10^3$	2	1

Modification of the original serum-free medium was attempted by substitution of the L-15 with Medium-199 with Hanks' salts or Eagle's MEM with Earle's salts. This substitution did not adversely affect the growth rate of cells. In fact, after four passages in the modified serum-free media, the cellular growth rates were higher than that in the original serum-free medium containing L-15. These findings suggest that the TRA-284-SF subline could be grown in serum-free media containing any of the basal media used for mammalian cell cultures. This should make it possible to select a more economical, useful, and/or convenient medium that suits the needs of investigators dealing with dengue virus replication in mosquito cells.

4.4. Identification of Dengue Viruses

4.4.1. Fluorescent antibody techniques for identification of dengue virus in infected tissues.

Fluorescent antibody (FA) tests are relatively rapid and simple and have been applied with some success to the diagnosis and study of the pathogenesis of dengue infection in fatal human cases or suspected cases of dengue hemorrhagic fever and to pathogenesis studies in laboratory animals. However, the diagnosis of dengue virus infection in fatal human cases of suspected dengue hemorrhagic fever is still difficult using serologic and present FA techniques, and time consuming using virus isolation methods.

A new indirect FA technique was evaluated for the detection of dengue virus antigen in infected mouse tissues. The biotin-avidin system (unlabeled antiviral antibody, biotinyl-anti-IgG and fluorescein conjugated avidin D) theoretically enhances the sensitivity of the FA method by amplifying the number of fluorescein particles attached indirectly to antigen.

Using antibody endpoint titers in dengue-infected suckling mouse brains as an assay for sensitivity, the biotin-avidin system was compared with the standard direct and two-step indirect FA techniques. Comparative tests were done on frozen sections of mouse brains with infectivity titers between 4.5 and 8.3 log $_{10}$ LLC-MK$_2$ cell PFU/g.

For the direct FA test, a conjugate prepared from a single human serum with a high titer of cross reactive flavivirus antibodies was used. As first antibody in the indirect FA and biotin-avidin test systems, hyperimmune mouse ascitic fluid (MAF) against dengue-2 virus was used. The second antibody in the IFA test was FITC-conjugated goat antiviral-IgG. Second and third antibodies for the biotin-avidin system were biotin goat antiviral IgG and FITC-conjugated avidin D, respectively. The optimal dilutions of second and third reagents were determined by prior tests using SLE virus in cell culture and infected mouse brains. Serial twofold dilutions of first antibody were added to the frozen sections to define the relative sensitivity of the three systems. Controls were normal mouse brains and infected mouse brains inoculated with normal MAF.

Brains from suckling mice incubated with dengue 2 virus were harvested at 24 intervals. One hemisphere was used for viral infectivity assays and the other for FA testing of frozen sections.

Table 4.17 shows the results of the endpoint titrations of antibody in mouse brain for the three FA systems. Comparison of the indirect FA and biotin-avidin systems with direct FA tests is not strictly appropriate because human antibody was used in the direct system, whereas hyperimmune MAF was used for both the indirect FA and biotin-avidin tests. The sensitivity determined by antibody endpoint titers, was highest for the biotin-avidin system, titers were 2- to 8-fold higher than those of the IFA system ($p \leq .01$). At optimal dilution, the intensity of fluorescence was greater with the biotin-avidin than with the direct FA or indirect FA system. In general, antibody titers did not vary greatly within FA test systems with change in tissue infectivity titer.

The results of this study provide evidence for an increased sensitivity of the biotin-avidin FA techniques for detecting dengue antigen in infected mouse brain tissue. Comparison of the three FA systems in tissues with infectivity titers lower than 4.5 log PFU/g would be important. It is possible that the biotin-avidin system can detect virus at lower concentrations than the one and two-step direct FA and indirect FA techniques. The biotin-avidin FA system may also be applicable to detection of viral antigen in formalin fixed tissues treated with trypsin and in lymphocytes.

4.4.2. FA staining of lymphocytes for rapid diagnosis of dengue viruses. Pathogenesis studies in Thailand have shown that dengue antigen can be detected on the surface of human lymphocytes by FA staining. We are attempting to develop this technique as a possible rapid diagnostic test in patients hospitalized with suspected dengue hemorrhagic fever.

Lymphocytes are separated from fresh plasma of suspected dengue patients and controls using a ficoll-hypaque gradient. Lymphocytes are then washed and placed on glass slides as spot smears. After fixing in acetone, the lymphocytes are stained by the direct FA method.

In preliminary work, lymphocytes from 4 patients in the third to sixth days of illness have been studied. In 2 patients, dengue 4 virus was subsequently isolated in tissue culture. In the other 2 patients, isolation has not yet been attempted. The FA staining of lymphocyte preparations from all 4 patients has been negative.

In future work, separated lymphocytes also will be incubated for 3-4 days and tested again by FA for the presence of dengue antigen. In addition, lymphocytes will be disrupted by sonic energy and inoculated into tissue culture and mosquitoes for virus isolation.

Table 4.17. Titer of Antibody by Three Fluorescent Antibody Techniques Applied to Detection of Dengue-2 Viral Antigen in Mouse Brain.

	Viral Infectivity Titer (log_{10} LLC-MK_2 PFU/g)							
	4.5	4.85	5.5	6.4	7.0	7.6	7.95	8.3
DFA	10	320	320	160-320	320	320	320	160-320
IFA	160	160	320	160	320	320	320	160
BA	1280	1280	640	640	640	1280	1280	1280

DFA = Direct Fluorescent Antibody Test.

IFA = Indirect Fluorescent Antibody Test.

BA = Biotin-Avidin Fluorescent Antibody Test.

4.4.3 Gas Liquid Chromatographic Analysis of Dengue Infected LLC-MK_2 Cell Cultures - GLC analysis of metabolic changes in LLC-MK_2 cell cultures infected with four serotypes of dengue viruses by frequency pulsed electron capture gas liquid chromatography. In the past several years, the use of gas-liquid chromatography (GLC) has been found useful in diagnosing some bacterial and viral diseases in man. As a preliminary phase of a study to investigate the possible application of the technique to dengue infection, metabolic changes in the dengue-infected cell cultures were studied jointly by J.B. Brooks of Bacterial Diseases Division and G. Kuno and R. B. Craven of Vector-Borne Diseases Division. Monkey kidney cell cultures (LLC-MK_2) were infected with four serotypes of dengue viruses, and the supernatant fluids of the cell cultures were extracted and derivatized for analysis by frequency pulsed electron capture gas-chromatography (FPEC-GLC) for amines, alcohols, carboxylic acids, and hydroxy acids. Supernatant fluids of uninfected cell cultures and a maintenance medium served as controls. Virus replication was studied by plague assay. History of the dengue viruses used in the experiment is shown in Table 4.18. The results showed that most of laboratory adapted viruses grew well, while low passaged strains did not replicate well (Table 4.19). Nevertheless, the different levels of viral replication shown in Table 4.19 did not affect FPEC-GLC analysis.

As shown in Fig. 4.2 (A,B), there occurred a dramatic change in the hydroxy acid components in the supernatant fluids of infected culture as compared with those of control culture. When the FPEC-GLC chromatograms for hydroxy acids among the supernatant fluids from the cultures infected with 4 dengue serotypes (Fig. 4.3 A,B,C,D) were compared with the control profiles (Fig. 4.2 A), it was evident that all profiles of infected cultures were different from that of the control. Furthermore, the profiles of the 4 serotypes differed among themselves. For example, 2 new peaks (N8a, N11a) found in the DEN 2 profile were not detected in the DEN 1 profile (Fig. 4.3). Similarly, the DEN 3 profile differed from the DEN 1 profile by the appearance of new peaks (N11a, N13) and an increased level of peak 4. DEN 3 differed from DEN 2 by the disappearance of peak N8a, an increased level of peak 9, and by the appearance of a new peak (N13). The most striking characteristics of the DEN 4 profile was (a) lack of metabolism of peak 8, (b) presence of a large amount of peak 9, and (c) the fact that only one new peak (N12) not found in the control profile is present. Thus, a combination of profiles of peaks 8 and 9 distinguish DEN 4 from DEN 1 or DEN 2. Further, DEN 2 and DEN 3 can be distinguished from DEN 1 or DEN 4 by the presence of peaks 8a, 11a, and 13.

The changes in amine profile that occurred during infection are demons- trated in Fig. 4.4. DEN 1 was the only serotype that produced a different profile from that of control. The significance of the reduction of peaks 1 and 5 in the DEN 1 profile (Fig. 4.4 A), as compared with the control profile (Fig. 4.4 B), was questionable since those peaks were absent in another control (Fig. 4.4 C) in which a different lot of growth medium was used.

Fig. 4.5 shows carboxylic acid profiles of normal and infected cultures. Profile differences among different lots of growth medium were again detected, as demonstrated by the lack of peaks 2 and 4 in lot 2. The profile of DEN 1 infected culture is clearly distinguished from that of the control by the appearance of new peaks (N3, N6) and the reduction of peaks (1, C4, 1C5). Fig. 4.6 shows that carboxylic acid profiles by all serotypes are different from that of controls because three peaks (U1, U2, U1C5) in the control were consistently reduced, and a new peak (N6) not detected in the control, was present. The DEN 1 profile for carboxylic acids was further distinguished from the profiles of the other serotypes by a new peak (N3).

Table 4.18. History of Dengue Virus Strains Used in the GLC Study.

Accession Number	Serotype	Virus Deisgnation and Passage History	Year Isolated	Place
CA1816	Dengue I	Hawaiian Prototype One monkey, one mosquito and Seven tissue culture passages	1944	Hawaii
CA1817	Dengue I	H-13806[a]/ Four tissue culture passages	1977	Jamaica
CA1818	Dengue I	H-23333[a]/ Four tissue culture passages	1977	Puerto Rico
CA1819	Dengue I	H-45509[a]/ Two tissue culture passages	1980	Mexico
CA1821	Dengue II	H-14241[a]/ Three tissue culture passages	1977	Puerto Rico
CA1822	Dengue II	H-20919[a]/ Two tissue culture passages	1977	Puerto Rico
CA1820	Dengue II	New Guinea "C" Prototype Twenty-four suckling mouse and Six tissue culture passages	1944	New Guinea
CA1823	Dengue III	H-87 Prototype One monkey and Nineteen tissue culture passages	1956	Philippines
CA1824	Dengue III	PR-6 Thirteen suckling mouse passages	1963	Puerto Rico
CA1825	Dengue III	H-21320 Two tissue culture passages	1977	Puerto Rico
CA1826	Dengue IV	H-241 Prototype Seven suckling mouse and Six tissue culture passages	1956	Philippines
CA2039	Dengue IV	H-54101 Two tissue culture passages	1981	Dominica
CA2040	Dengue IV	H-54157 Two tissue culture passages	1981	Saint Barthelem

[a]/Virus identification number at the San Juan Laboratories.

Table 4.19. Replication of dengue virus strains used in FPEC-GLC analysis.

Virus	Inoculum (Log PFU[a]/Flask)	Extracellular virus titer (Log PFU[a]/ml supernatant fluid) 7 days after inoculation
DEN 1 (Hawaii)	3.6	5.9
DEN 1 (H-13806)	3.4	3.5
DEN 1 (H-23333)	3.9	2.8
DEN 1 (H-45509)	3.6	3.2
DEN 2 (New Guinea "C")	3.6	6.8
DEN 2 (H-14241)	3.5	3.4
DEN 2 (H-20919)	3.1	2.9
DEN 3 (H-87)	3.3	4.3
DEN 3 (PR-6)	3.9	3.7
DEN 3 (H-21326)	3.8	2.7
DEN 4 (H-241)	3.6	4.5
DEN 4 (H-54101)	4.0	5.8
DEN 4 (H-54157)	4.0	6.8

a/ PFU: plaque forming unit

Fig. 4.2 A comparison of frequency pulsed electron capture gas-liquid chromatography (FPEC-GLC) chromatograms for hydroxy acids in the supernatant fluids of control and dengue-infected LLC-MK_2 cell cultures.

Column: OV-101.

Abbreviations

TC: tissue culture; MK; LLC-MK_2 cells; HYD: heptafluorobutyric anhydride-ethanol derivatized acidic diethyl ether extracts; reag: reagent; LAC: lactic acid; 2-OH But: 2-hydroxybutyric acid; IS: internal standard; 2-OH Val; 2-hydroxy valeric acid. "U" over a peak indicates utilized; "N" over a peak indicates a new peak.

Figure 4.2

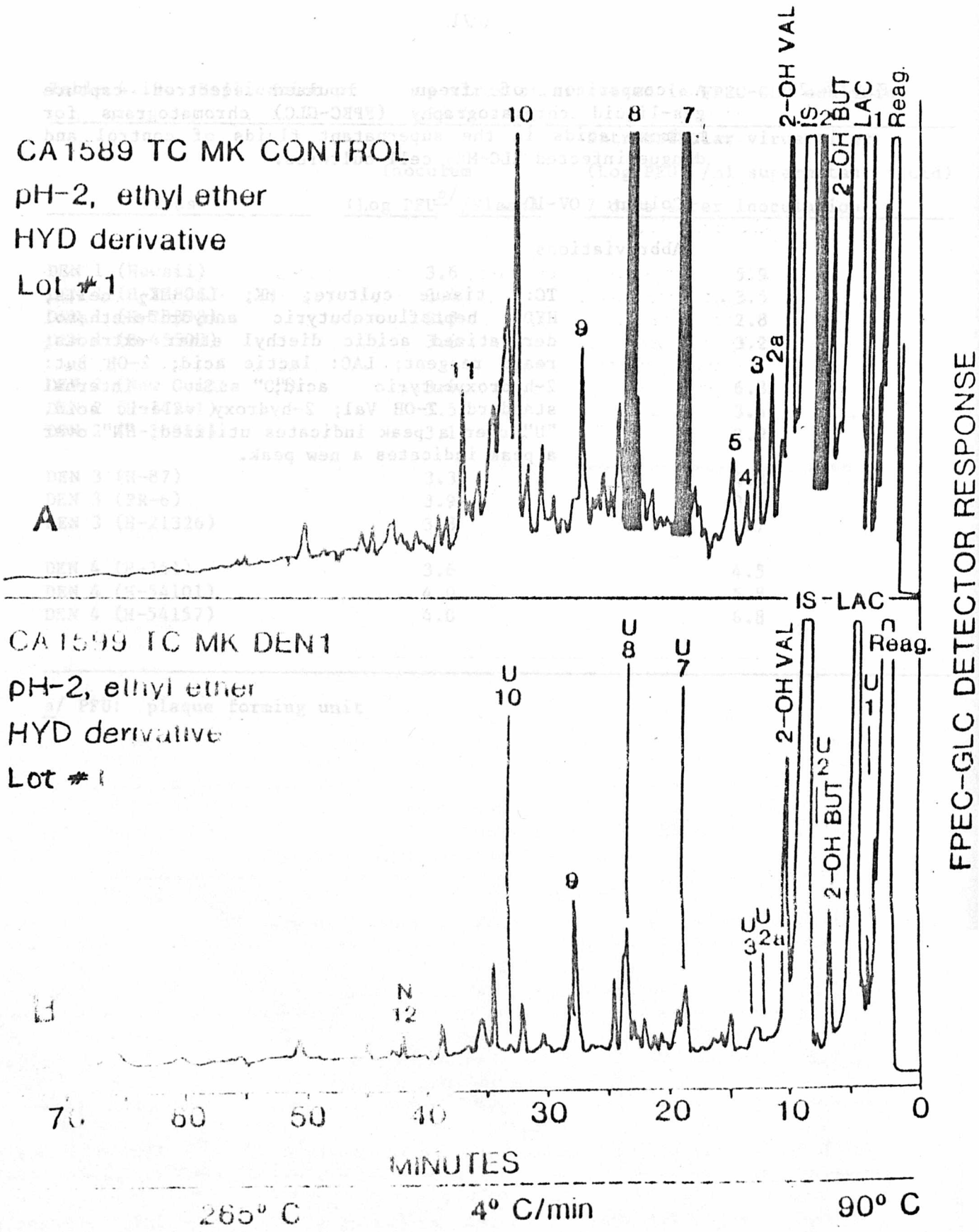

CA1589 TC MK CONTROL
pH-2, ethyl ether
HYD derivative
Lot #1
A
11
10
9
8
7
5
4
3
2a
2-OH VAL
IS
2
2-OH BUT
LAC
1
Reag.
IS - LAC
CA1589 TC MK DEN1
pH-2, ethyl ether
HYD derivative
Lot #1
B
N
12
U
10
9
U
8
U
7
U
3
U
2a
2-OH VAL
2-OH BUT
U
2
U
1
Reag.
FPEC-GLC DETECTOR RESPONSE
70
60
50
40
30
20
10
0
MINUTES
265° C
4° C/min
90° C

Fig. 4.3 A comparison of FPEC-CLC chromatogram for hydroxy acids in the supernatant fluids of LLC-MK_2 cell cultures infected with 4 dengue serotypes.

Column: OV - 101

Abbreviations

TC: tissue culture; MK; LLC-MK_2 cells; HYD: heptafluorobutyric anhydride-ethanol derivatized acidic diethyl ether extracts; reag: reagent; LAC: lactic acid; 2-OH But: 2-hydroxybutyric acid; IS: internal standard; 2-OH Val; 2-hydroxy valeric acid. "U" over a peak indicates utilized; "N" over a peak indicates a new peak.

Figure 4.3.

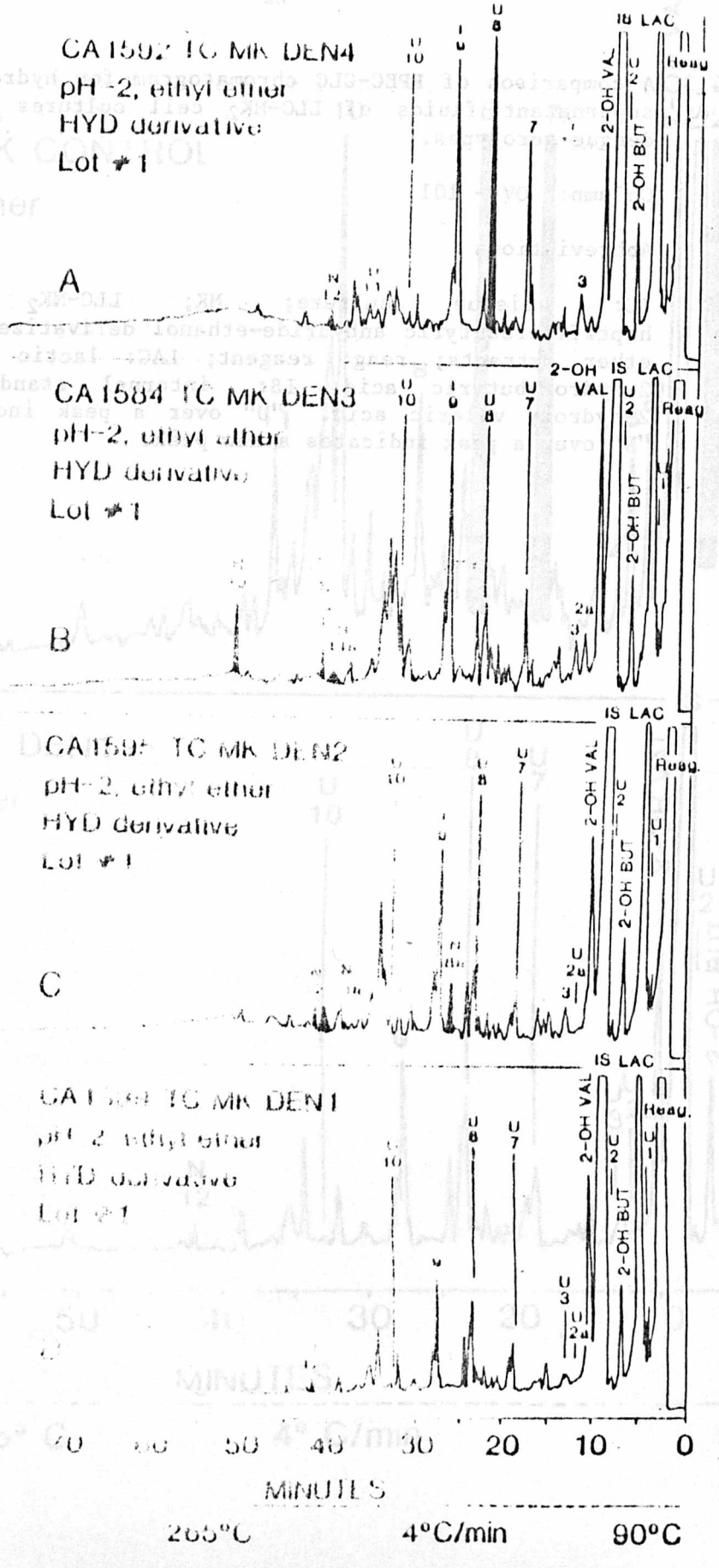

A
B
C
2-OH VAL
2-OH BUT
IS LAC
Reag.
FPEC–GLC DETECTOR RESPONSE
MINUTES
265°C
4°C/min
90°C

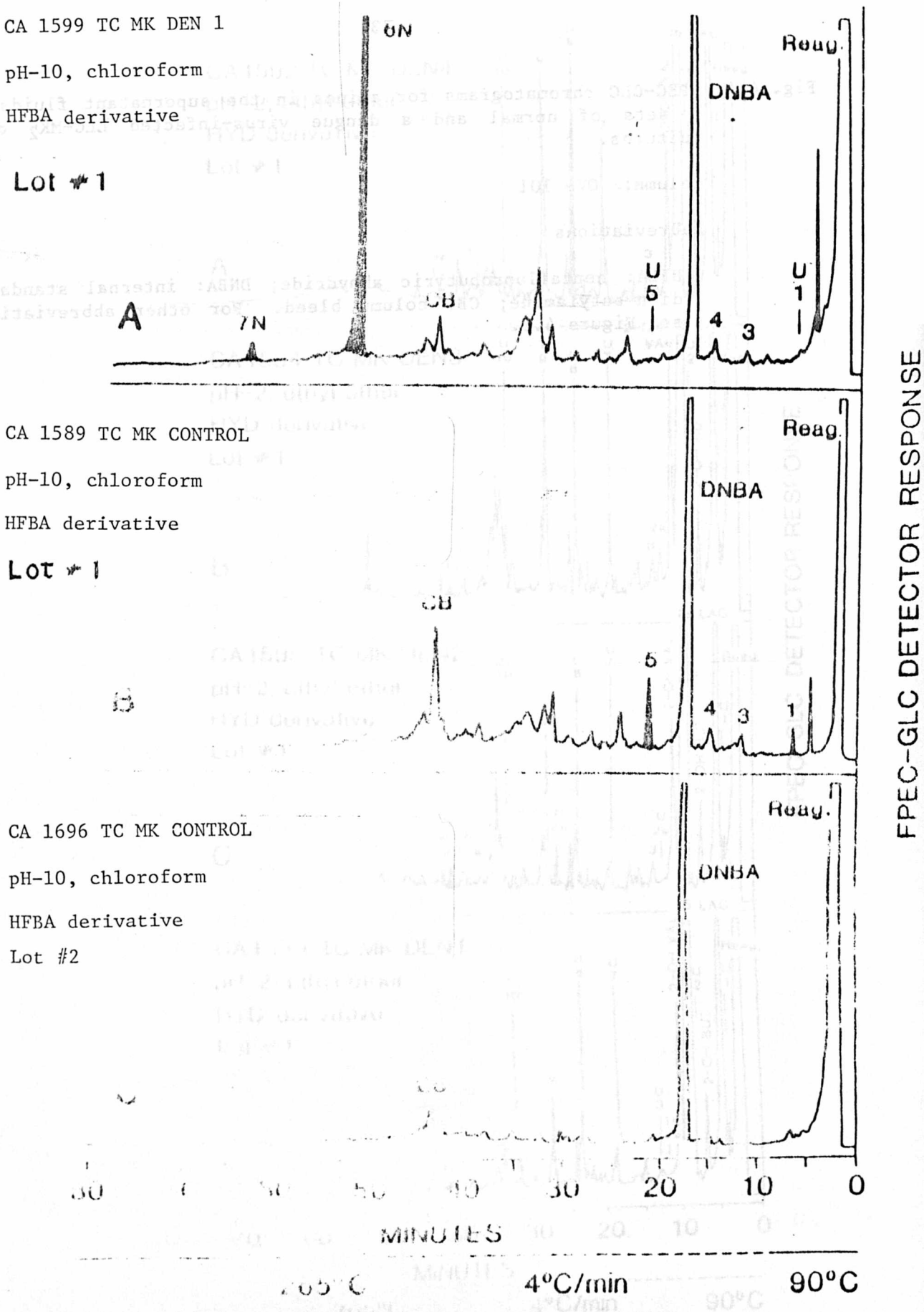
Fig. 4.4 FPEC-CLC chromatograms for amines in the supernatant fluids of 2 sets of normal and a dengue virus-infected LLC-MK_2 cell cultures.

Column: OV- 101

Abbreviations

HFBA: heptafluorobutyric anhydride; DNBA: internal standard, di-n-butylamine; CB: column bleed. For other abbreviations see Figure 4.2.

Figure 4.4.


CA 1599 TC MK DEN 1
pH-10, chloroform
HFBA derivative
Lot #1
6N
Reag.
DNBA
A
7N
CB
U
5
4
3
U
1
CA 1589 TC MK CONTROL
pH-10, chloroform
HFBA derivative
Lot #1
Reag.
DNBA
CB
5
4
3
1
CA 1696 TC MK CONTROL
pH-10, chloroform
HFBA derivative
Lot #2
Reag.
DNBA
30
20
10
0
MINUTES
4°C/min
90°C
FPEC-GLC DETECTOR RESPONSE

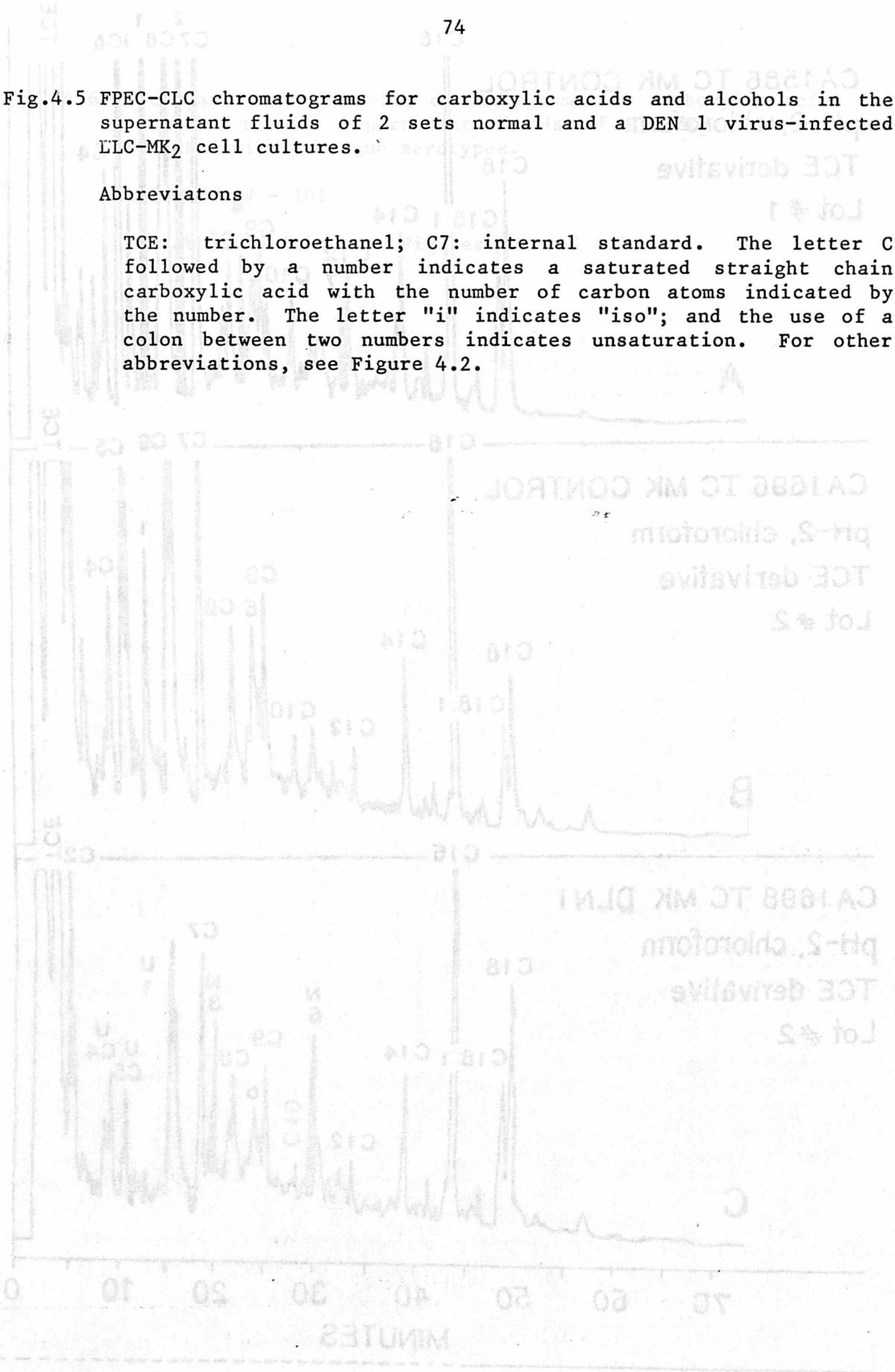


Fig.4.5 FPEC-CLC chromatograms for carboxylic acids and alcohols in the supernatant fluids of 2 sets normal and a DEN 1 virus-infected LLC-MK_2 cell cultures.

Abbreviatons

TCE: trichloroethanel; C7: internal standard. The letter C followed by a number indicates a saturated straight chain carboxylic acid with the number of carbon atoms indicated by the number. The letter "i" indicates "iso"; and the use of a colon between two numbers indicates unsaturation. For other abbreviations, see Figure 4.2.

FIGURE 4.5

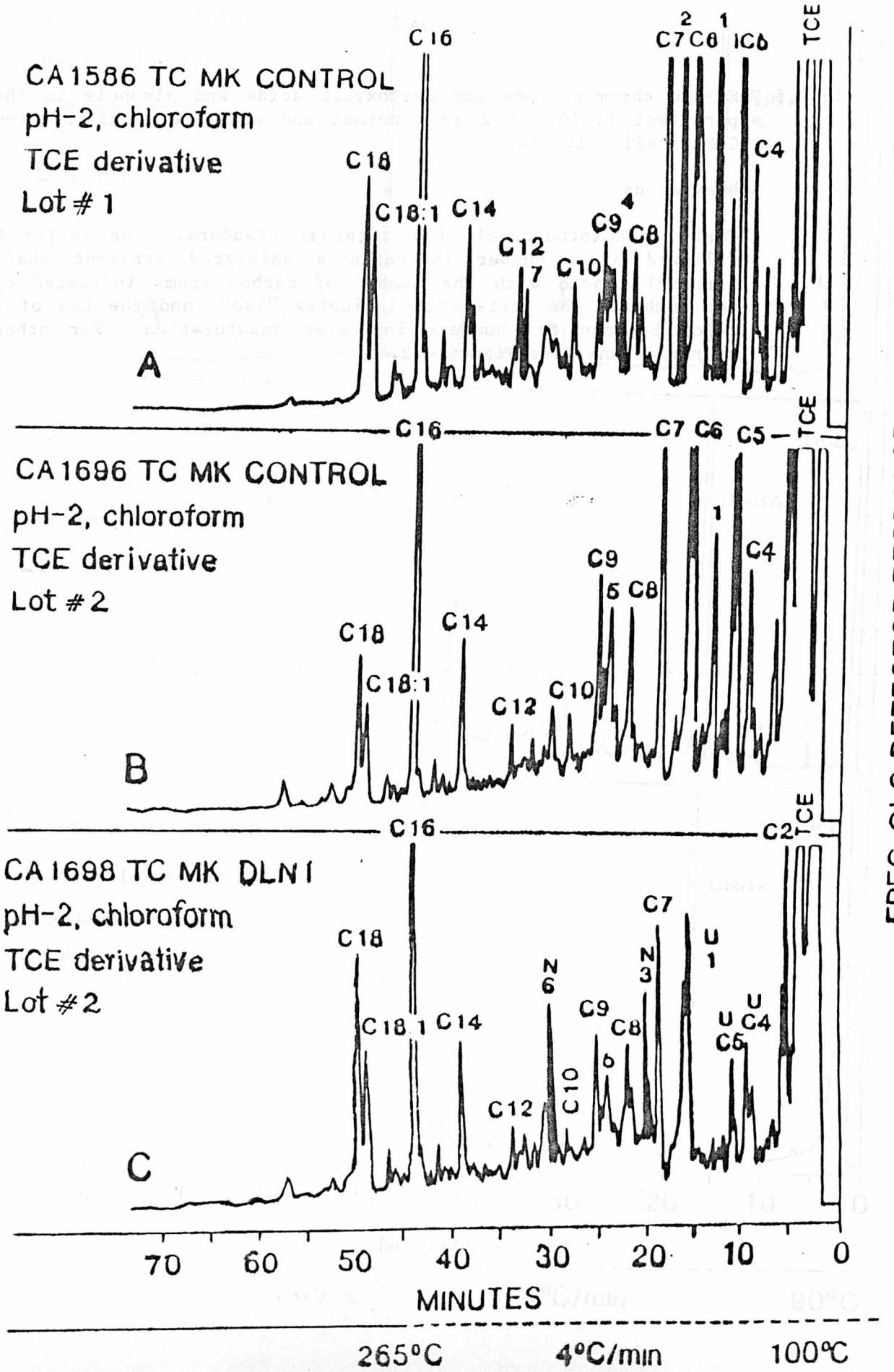
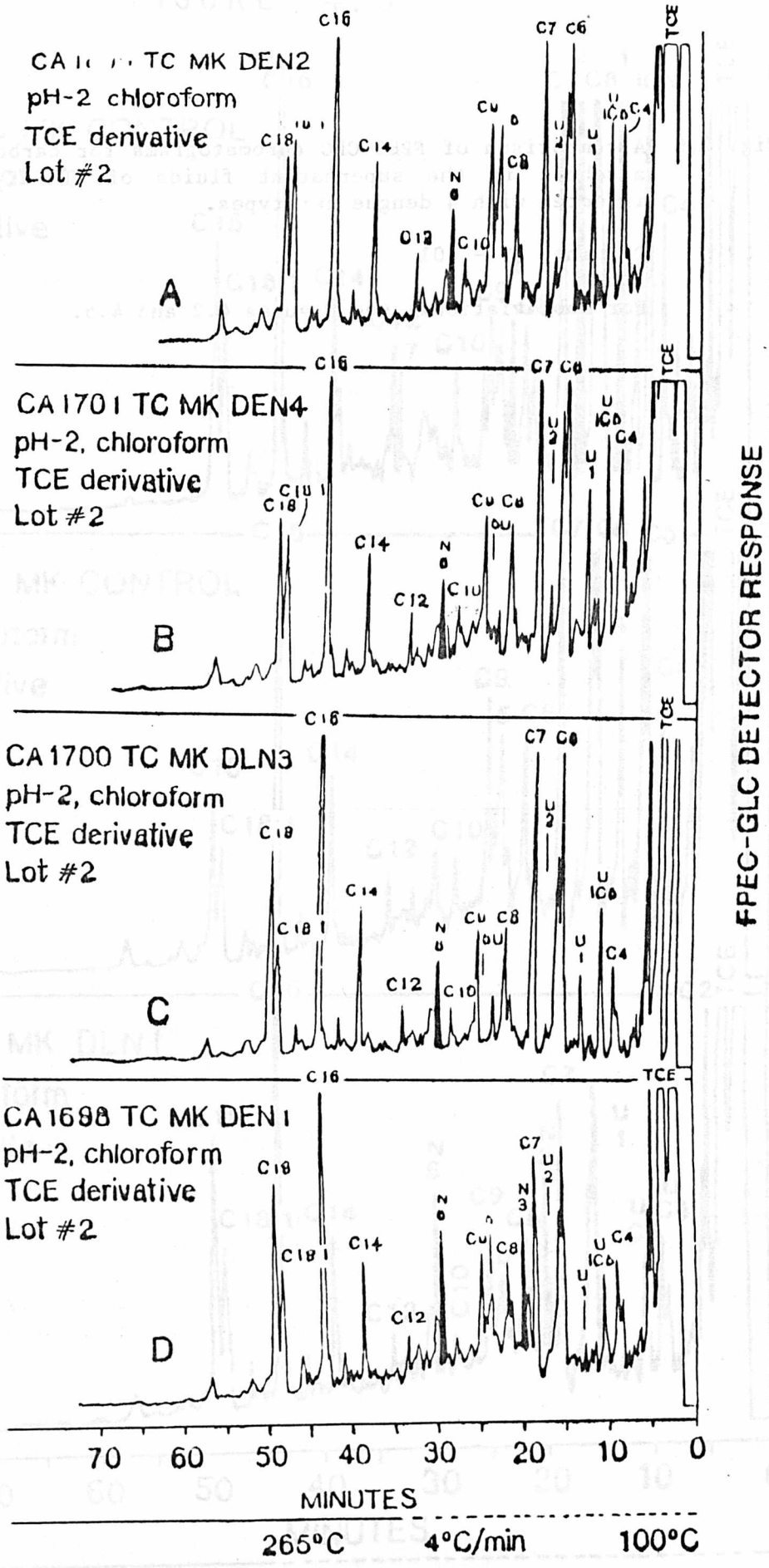

CA1586 TC MK CONTROL
pH-2, chloroform
TCE derivative
Lot # 1
A
C16
C18
C18:1
C14
C12
C10
C9
C8
C7
C6
C4
TCE
CA1696 TC MK CONTROL
pH-2, chloroform
TCE derivative
Lot # 2
B
C16
C18
C18:1
C14
C12
C10
C9
C8
C7
C6
C5
C4
TCE
CA1698 TC MK DLN1
pH-2, chloroform
TCE derivative
Lot # 2
C
C16
C18
C18:1
C14
C12
C10
C9
C8
C7
C6
C5
C4
C2
TCE
70
60
50
40
30
20
10
0
MINUTES
265°C
4°C/min
100°C
FPEC-GLC DETECTOR RESPONSE

Fig. 4.6 A comparison of FPEC-CLC chromatograms for carboxylic acids and alcohols in the supernatant fluids of LLC-MK_2 cell cultures infected with 4 dengue serotypes.

Column: OV - 101

For abbreviations, see Figures 4.2 and 4.5.

FIGURE 4.6


CA 1 TC MK DEN2
pH-2 chloroform
TCE derivative
Lot #2
A
CA1701 TC MK DEN4
pH-2, chloroform
TCE derivative
Lot #2
B
CA1700 TC MK DLN3
pH-2, chloroform
TCE derivative
Lot #2
C
CA1698 TC MK DEN1
pH-2, chloroform
TCE derivative
Lot #2
D
C16
C18
C14
C12
C10
C8
C7
C6
C4
TCE
70
60
50
40
30
20
10
0
MINUTES
265°C
4°C/min
100°C
FPEC-GLC DETECTOR RESPONSE

4.5. Production of Toxorhynchites amboinensis

A consistent daily production of adult Toxorhynchites amboinensis is essential for the clinical and virologic studies of the Dengue Branch. Initial production of this species was erratic at San Juan Laboratories. The purpose of this effort is to increase and stabilize daily adult production at 100 individuals (600 adult/week) and to minimize technician time.

Initial investigations were involved with daily scheduling based on developmental rates of Tx. amboinensis and Aedes aegypti. Originally, Tx. amboinensis were reared using 2 methods, open plastic pans and containers with 24 separate compartments for single specimen rearing. Due to prolonged developmental time exhibited by Tx. amboinensis reared in individual compartments (20-22 days egg to pupae versus 14-15 days in open pans), plus the time consuming effort required for daily maintenance, the individual rearing procedure was eliminated.

In order to provide adequate food for the Tx. amboinensis larvae at least 20,000 Ae. aegypti larvae must be hatched per week. This provides enough first and second instar food for young Tx. amboinensis as well as third instar for the older Tx. amboinensis. The Ae. aegypti used for food are reared in large plastic pans with approximately 2000/pan for 3 days. On day 4, the larvae are removed and placed in aliquats of 5000 in a 500 ml container and put in an environmental chamber at 15° C until needed. Using this method, a backlog of Ae. aegypti can be stored for 3 days before use.

Preliminary experiments indicated that a maximum of 400 newly hatched Tx. amboinensis should be set up per day to achieve the target number of adults. This number was established based on survivorship and the feeding capability of the laboratory. Initially, the survivorship ranged from 31 to 51 percent. However, it has increased to 60-68 percent, primarily due to 2 changes in procedure. First, it was found that during the first 7 days Tx. amboinensis requires high densities of Ae. aegypti not only for food, but as a buffer which inhibits aggression and cannibalism. After 7 days this behavior diminishes such that less than 1-2 percent of the total mortality occurs during the duration of the larval stage. Second, newly emerged Tx. amboinensis larvae are placed in enamel pans (50/pan) which are filled with approximately 250 ml. water and slanted so that only 1/4 of the pan is covered with water. This procedure plus the addition of 5000 Ae. aegypti first or second instars every other day for 4 days decreased mortality from 30% to 10%. It was calculated that slanting the pan accounted for a decrease of 12% in mortality. These new findings are now being incorporated into the routine schedule.

The Ae. aegypti colony strain was also changed. The ROCK strain of Ae. aegypti is now being used because of its fast developmental rate and increased fecundity. Because of this fewer females are needed to provide adequate numbers of eggs, thus space and maintenance time are reduced.

Two ongoing studies with Tx. amboinensis which may aid in increased yield and decreased maintenance time are: (1) to determine if Tx. amboinensis eggs can be stored for a significant period of time and (2) whether the cannibalistic behavior of this species can be decreased by selective breeding. To date, Tx. amboinensis eggs have been held on moist filter paper at 15° C for 9 days. The percent survival of 3 experiments each with 2 petri plates (25 eggs/plate) was 96%. This procedure also results in a more synchronous hatch when the eggs are placed on water.

Preliminary experiments with pairs of Tx. amboinensis in the presence of high densities of Ae. aegypti has revealed a low frequency of individuals which show no aggressive tendencies. In each test the Tx. amboinensis larvae are similar in age and size and the number of Ae. aegypti present and/or eaten daily are known. This inbreeding experiment is now in the third generation with the non-aggressive behavior increasing from 2%/200 larvae in generation 1 to 10%/200 larvae in generation 2.

5. PUBLICATIONS

5.1. Published in 1981

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12. Tan, R.; Abdin, C.; Maroef, C. and Gubler, D.J. Comparative growth of dengue viruses in Aedes aegypti and Aedes albopictus after parental infection, Mosquito News, 41(1), pp 71-74, 1981.

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1. Gubler, D.J.; Novak, R. and Mitchell, C.J. Arthropod vector competence-epidemiological, genetic, and biological considerations (In Press)

2. Kay, B.H., Miles, J.A.R.; Gubler, D.J. and Mitchell, C.J. Vectors of Ross River virus: An overview. Proceedings International Seminar on Viral Diseases in South-East Asis and the Western Pacific, Canberra, Australia, February 1982 (In Press)

3. Kuno, G. Persistent infection of a nonvector mosquito cell line (TRA-171) with dengue viruses. Intervirology (In Press)

4. Mitchell, C.J. and Gubler, D.J. Variation in infectivity of St. Louis encephalitis viral strains for Culex pipiens quinquefasciatus and correlation with virulence markers in vertebrates, Am. J. Trop. Med. Hyg. (Submitted for publication)

5. Waterman, S.H. and Monath, T.P. Fluorescent Antibody Techniques Applied to the Identification of Dengue Viruses Infected Tissues, Acta Virologica (Submitted for publication)